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The Genetic Diversity of Endangered Fishes in the Northwest Atlantic

A Dissertation Presented

by

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Abstract of the Dissertation

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Genetic diversity is the fundamental building block of biodiversity, harboring the adaptive potential of populations. The magnitude of the two main factors eroding genetic diversity, inbreeding and genetic drift, increases as the effective population size (N_e) decreases. N_e describes the number of reproductively mature adults effectively contributing to the next generation. In populations with overlapping generations, the effective number of breeders (N_b). Loss of genetic diversity has long been a concern for terrestrial and freshwater organisms, but traditionally less so for fisheries management due to the paradigm that marine fish populations are large, panmictic and thus resistant to the erosion of genetic diversity. Therefore, despite widespread concern about the loss of marine fish biodiversity, there has been little concern regarding the genetic biodiversity. However, recent studies have discovered that many marine populations are in fact genetically structured and N_e is frequently several orders of magnitude smaller than census population size. Thus, I assessed the genetic diversity, N_e/N_b and levels of inbreeding in four populations of threatened or endangered species under the hypotheses that the genetic diversity of marine fish populations may be eroding as quickly as in some terrestrial animals and that in extreme cases inbreeding may occur. Indeed, evidence of genetic bottlenecks, low N_e/N_b and inbreeding was found in all four species: The Key silverside inhabits small, tidally restricted hypersaline pools. Extreme shifts in allele frequencies over 10+ generations and loss of genetic diversity demonstrate that the colonization of inland habitats resulting in fragmented populations has a significant impact on the genetic diversity of marine fish. Evidence of severe inbreeding was detected in winter flounder, demonstrating that even historically common marine fish can be prone to inbreeding as populations decline. First estimates of the effective number of breeders N_b for three spawning populations of Atlantic sturgeon were determined using juvenile fish captured at aggregation sites in New York Bight. DNA profiling of white sharks demonstrates their heightened vulnerability due to genetic factors. In conclusion, I place these studies within the context of a literature review of the genetic health of marine fish populations and suggest new avenues for research and standard methods for assessing genetic diversity in marine fishes.

To my Dad

Thank you for your unwavering love and support

To my Mom

Thank you for teaching me to see the beauty of creation

To the Big Guy upstairs

I lift my eyes up to the mountains...

Counting fish is like counting trees, except they are invisible and they keep moving.

John Shepherd

One fish, two fish, red fish, blue fish.

Dr. Seuss

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Preface

Small populations face a multitude of challenges, not the least of which are increased susceptibility to inbreeding (i.e. mating between related individuals) and the stochastic loss of genetic diversity. The former can directly reduce the fitness of individuals within the population (“inbreeding depression”), while the latter can reduce the adaptive potential of the population or species. Populations can lose genetic diversity if they experience a sudden and dramatic reduction in size (“bottleneck”) but also do so naturally during the transmission of genetic diversity between generations due to stochastic shifts in allele frequencies during recombination (“genetic drift”). Genetic drift is inversely proportional to the effective population size (N_e), which is the number of reproductively mature adults effectively contributing to the next generation and conceptually defined as the number of individuals in an idealized Fisher-Wright population experiencing the same amount of genetic drift as the study population. In a population with overlapping generations, the effective number of breeders (N_b) describes the number of reproductively mature adults that produced offspring during a certain time period. Recent analytical advances have enabled accurate estimation of the effective number of breeders during a given time period in small populations (< 500-1,000 adults), a parameter that can approximate the number of breeding individuals within a given time frame.

Populations of endangered species are, by definition, small and therefore inbreeding, therefore genetic drift and bottleneck effects are frequent issues of management concern. These concerns are especially acute for terrestrial and freshwater organisms, but traditionally less so for the management of marine fish. The overarching paradigm of marine fisheries management has traditionally been that populations are panmictic, large and thus resistant to the erosion of genetic diversity. These characteristics also dilute the risk of inbreeding. Hence, despite widespread concern about the loss of marine fish biodiversity in terms of species extinction and population extirpation, there has been much less concern about the trajectory of genetic biodiversity of marine fish populations. Additionally, genetic approaches have only recently been used to estimate N_b because previously it was assumed that marine fish populations are too large for these methods to be accurate. Overlooking these methods has potentially robbed fisheries managers of a means to estimate N_b and N_e , which is a critical parameter for conservation and management plans.

Even though many marine fish populations are known to be in decline due to exploitation they are still thought to be large and mixed compared to terrestrial or freshwater organisms. This paradigm is flawed for several reasons. Genetic studies have shown that many marine populations are far more structured than previously assumed, which is thought to be due to processes such as natal homing and larval retention. Broadcast spawners also have high variance in reproductive success, which means that N_b can be orders of magnitude smaller than census population size (N_c). The genetic diversity of marine fish populations may therefore be eroding as quickly as in some terrestrial animals. In extreme cases, marine fish may even be experiencing high levels of inbreeding. In light of these developments, I assessed the genetic diversity, N_e and levels of inbreeding in populations of marine fishes that are considered threatened or endangered. These species are *Acipenser oxyrinchus* (Atlantic sturgeon), *Carcharodon carcharias* (white shark), *Menidia peninsulae/conchorum* (tidewater and Key silversides) and *Pseudopleuronectes americanus* (winter flounder). Evidence of genetic bottlenecks, low effective population size and/or inbreeding was found in all of these species, confirming that these issues should also be routinely considered in the management of marine fish. My dissertation concludes with a

literature review of the genetic health of marine fish populations and suggestions of new avenues for research and monitoring.

In Chapter 1, I developed novel microsatellite loci for two of my study species, white sharks, *Carcharodon carcharias* and winter flounder, *Pseudopleuronectes americanus*. These markers will enhance studies of genetic diversity, fine scale population structure, N_e and mating behavior. Chapter 2 focuses on the characterization of genetic diversity and estimation of N_e in isolated populations of a marine fish, *Menidia conchorum*, which has made incursions into inland habitats (small hypersaline pools in the Florida Keys). I document extreme shifts in allele frequencies over 10+ generations, low N_e , as well as evidence for loss of genetic diversity, inbreeding and recent or ongoing bottlenecks. I conclude that the colonization of inland habitats resulting in fragmented populations can have significant impacts on the genetic diversity of marine fish. In Chapter 3, I show evidence for both severe inbreeding and small N_b in a formerly abundant marine fish, *Pseudopleuronectes americanus* in 6 Long Island bays. I conclude that even historically common marine fish can be prone to inbreeding as the population declines, a factor that should be considered in fisheries management and conservation plans. In Chapter 4, I assess N_b for the Hudson, Delaware Rivers and James River spawning populations of *Acipenser oxyrinchus*. These were estimated using juvenile fish captured at marine aggregation sites of subadults in the New York Bight. All three rivers also exhibit a genetic signature of a recent or ongoing bottleneck and are each composed of $N_b < 200$ effective breeders. These estimates were comparable to the estimated number of spawning adults in each river based on non-genetic method. This suggests that genetic estimates of N_b could be a useful tool for assessing spawning population size in other endangered fish with similar life-history characteristics. In Chapter 5, I investigate patterns of genetic diversity in two major white shark populations, the Northwest Atlantic and southern Africa. The populations proved to be genetically isolated from one another and all other previously surveyed white shark populations. There was strong evidence of recent population bottlenecks and contemporary inbreeding. The effective number of breeders in the Northwest Atlantic was estimated to consist of approximately 20-48 individuals. These results demonstrate that this marine apex predator exhibits characteristics comparable to small, fragmented populations of threatened terrestrial and freshwater species. In the final chapter, I assess the genetic health of marine fishes by reviewing the currently available literature on N_e estimates and evidence of inbreeding in marine fishes. I found that single-sample estimates of N_e in marine fish have dramatically increased, with almost 80% published in the last five years and encompassing 25 species. I attribute this to the availability of new estimators and a loosening of the paradigm that marine fish populations have N_e that are too high to measure using these approaches. Marine fish N_e estimates ranged from 10 to $>10^6$ individuals, 10% of study populations had point estimates below which inbreeding is a concern (<50 individuals), a further 21% were in danger of losing adaptive potential (50-500 individuals) and only 21% of study populations had sufficient effective population sizes to maintain long-term genetic stability. Though I identified only five studies reporting evidence of inbreeding in marine fish, I found that commonly reported deviations from Hardy-Weinberg Equilibrium (HWE) due to heterozygote deficiencies rarely result in further investigation of the potential role of inbreeding. My review indicates that low effective population size and inbreeding are valid concerns for marine fish conservation and fisheries management.

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None of these studies would have been possible without many people contributing tissue samples and allowing me to piggy-back on their projects. I would like to thank Keith Dunton for all his help with the sturgeon samples, Lyndie Hice, Mike Frisk and Anne McElroy for making me a part of the winter flounder project, Chris Martinez for his help with morphometrics and Hannes Bauman and Debra Abercrombie for their help sampling silversides.

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Chapter 1

Development of novel microsatellite loci for winter flounder, *Pseudopleuronectes americanus* and white sharks, *Carcharodon carcharias*

Introduction

Microsatellites are highly variable molecular markers that are useful for studies on an individual to population level. They are characterized by short tandem repeats (STRs), a motif of 1-6 basepairs that is repeated a variable number of times. This results in alleles which differ only in the number of repeat units. Microsatellites are non-coding, selectively neutral and found throughout the eukaryote genome. Once microsatellite loci have been isolated for a particular species they can be amplified through PCR using primers designed to bind to the flanking regions of the locus. Due to Mendelian inheritance, inferences can be made regarding the relationships between sampled individuals. Thus, they are commonly used to determine kinship of individuals and pedigrees using DNA fingerprinting. Furthermore, they are the molecular marker of choice to analyze demographic parameters of populations including migration patterns and population structure, genetic diversity, levels of inbreeding, effective population size and the occurrence of bottlenecks. Here, I report the development of 22 novel species-specific loci for winter flounder, *Pseudopleuronectes americanus*, and ten loci for white sharks, *Carcharodon carcharias*.

Winter flounder is a commercially and recreationally exploited flatfish that inhabits the inshore bays and estuaries of the Northwest Atlantic coast from Labrador to Georgia (Buckley 1989, Hendrickson et al. 2006, McCracken 1963). It is currently managed as three designated population segments (DPS) in the United States (the Gulf of Maine, New England/Mid Atlantic Bight and Georges Bank) though there are indications of localized fine-scale population structure (Hendrickson et al. 2006, McClelland et al. 2005). Winter flounder began declining in the late 1980s and commercial (NMFS 2007) and recreational landings (<http://www.st.nmfs.noaa.gov/recreational-fisheries/access-data/run-a-data-query/index>) have reached record low harvest levels. Despite management effort, there is little evidence of recovery (Hendrickson et al. 2006). Previous population genetic studies have used a small number of

flatfish microsatellite loci to delineate population structure (Crivello et al. 2004, DeCelles et al. 2007, McClelland et al. 2005), though none have used loci isolated specifically from the winter flounder genome. Additional microsatellite loci would enhance the accuracy of estimations of population differentiation, effective population size, mating behavior and genetic diversity.

The white shark is one of the largest predatory fish in the world and is widely distributed in temperate and subtropical waters (Compagno et al. 1997). Significant research efforts have been made toward understanding white shark movements, both to improve conservation of this relatively rare species and to elucidate its ecological role as an apex predator (Bonfil et al. 2005, Joergensen et al. 2010). Even though white sharks are capable of oceanic migration, population genetic studies based on sequencing of the mitochondrial control region have demonstrated genetically distinct populations occur off the coast of California (Joergensen et al. 2010), South Africa (Pardini et al. 2001), Australasia (Pardini et al. 2001, Blower et al. 2012) and Japan (Tanaka et al. 2011). Only two studies also used nuclear microsatellites to examine genetic population structure, one of which was discordant with mitochondrial results and has been widely cited as evidence of sex-biased dispersal in this species (Pardini et al. 2001, Blower et al. 2012). Both studies relied on a small number of loci that have low to moderate polymorphism. Additional loci with greater polymorphism would increase the power to detect differentiation in the nuclear genome and enable more detailed studies of white shark population structure and breeding biology.

Primer note

Genomic DNA was isolated from an individual winter flounder sampled in Shinnecock Bay, New York, using the Qiagen Blood and Tissue extraction kit (Qiagen, Valencia, California, USA) and from white sharks sampled in the US Atlantic. A microsatellite library was developed from this DNA sample using an enrichment protocol, which employs streptavidin-coated magnetic beads and biotin-labeled repetitive probes (Glenn & Schable 2005) to create bacterial clones containing microsatellites. Primers were designed from cloned sequences containing a microsatellite. DNA sequencing was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California), and sequences were run on an ABI 3730 DNA Analyzer. We developed primers flanking core microsatellite repeats using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primer pairs were optimized for locus-specific annealing temperatures and MgCl₂ concentrations (Table 1-1, 1-2, 1-3). Final

reaction volume of 10 μ l contained 1x PCR buffer, 10x bovine serum albumin, 1.5 – 2.5 mM MgCl₂, 0.12 mM each of dNTP, 0.16 μ M reverse primer and fluorescently labeled M13 primer, 0.04 μ M species-specific forward primer and 1 unit of Taq polymerase. Thermal cycling profiles were 4 min at 94°C, 30 cycles of 94°C for 15 seconds, primer specific annealing temperature T_a for 15 seconds, 72°C for 45 seconds and 5 cycles of 94°C, 15 seconds at 53°C and 45 seconds at 72°C with a final extension for 10 minutes at 72°C.

PCR products were amplified from genomic DNA templates extracted from 0.015 – 0.035g of fin tissue obtained from individual winter flounder captured in Shinnecock Bay (N=67) and US Atlantic white sharks (N=23). PCR products were separated and sized using an internal size standard (LIZ-500 Applied Biosystems) on a Genetic Analyzer (Applied Biosystems ABI3730 sequencer) and scored using ABI PeakScan v10. FSTAT (Goudet 1995) was used to determine the genetic diversity of the analyzed individuals for both species.

For winter flounder 22 loci were successfully amplified, nine of which were polymorphic with the number of alleles observed ranging from 6 – 56 (Table 1-1). The remaining loci were monomorphic for Shinnecock Bay (Table 1-2). The observed and expected heterozygosities of the polymorphic loci ranged from 0.092 – 0.69 and 0.161-0.954, respectively (Table 1-1). No evidence of stuttering or large allelic dropout was detected using MICROCHECKER (van Oosterhout et al. 2004). We tested for linkage disequilibrium and Hardy-Weinberg equilibrium (HWE) with the exact tests implemented in GENETPOP (Raymond & Rousset 1995; Rousset 2008). No linkage disequilibrium was detected, though all polymorphic loci with the exception of WF06 showed significant deviations from HWE caused by excess homozygotes.

This pattern is most likely to have a biological explanation, such as inbreeding, rather than null alleles given that it occurs at all of the loci rather than just a subset of them. Three additional observations support this idea. First, all samples amplified at all loci, which means there are no null homozygotes. This would be highly unlikely if the large excess of homozygotes observed were due to null alleles. Second, we reamplified 8 homozygotes for each locus at lower annealing temperatures (up to 3°C lower than the T_a given in Tables 1-1 and 1-2) to reveal null alleles at lower stringency. None were observed. Lastly, we amplified these same samples with other published primer sets where no heterozygote deficiencies were detected (A441, J42 (Crivello et al. 2004), Pam21, Pam27, Pam79 (McClelland et al.2005). All of them exhibited similar strong heterozygote deficiencies in the study population (F_{IS} = 0.159-0.367). Winter

flounder are at record low levels in Long Island estuaries due to overexploitation (Hendrickson et al. 2006) and we hypothesize that these deviations are due to inbreeding, similar to some freshwater fish, e.g. char (Castric et al. 2002) and heavily exploited marine flatfish (Hoarau et al. 2004).

For the white sharks 10 loci were identified with 2 -13 alleles per locus and expected and observed heterozygosity ranged between 0.29-0.849 and 0-0.744, respectively (Table 1-3). MICROCHECKER (van Oosterhout et al. 2004) did not detect evidence for stuttering or large allelic dropout. Exact tests of Hardy-Weinberg Equilibrium (HWE) and linkage disequilibrium were calculated as implemented in GENEPOP (Raymond & Rousset 1995, Rousset 2008). No evidence for linkage disequilibrium was detected. Deviations from HWE were detected for 5 loci, due in all cases to significant heterozygote deficiency. We re-amplified 8 homozygotes for all loci at ($T_a - 3^\circ\text{C}$). Despite primers annealing less specifically at lower temperatures no additional alleles were detected, suggesting that null alleles might not explain these deviations. Analysis of the same 5 loci in white sharks from South Africa (N=27) did not exhibit the same deviations from HWE for some of these loci (CCa9, CCa1226, CCa1273), again suggesting a biological rather than technical explanation for these deviations. When combined with other microsatellite loci available for these species (Pardini et al. 2001, Feldheim et al. 2007), the novel loci presented here will enable higher resolution population genetic studies of these two threatened sharks, as well as opening up the possibility of doing comprehensive studies of individual relatedness and breeding biology.

Table 1-1: Characterization of polymorphic microsatellite loci developed from genomic library for winter flounder, *Pseudopleuronectes americanus*.

Locus	Primer sequences	repeat	T_a	[MgCl₂]	bp	A	Ho	He	GB no.
WF06	WF06-F: ATCAGGGGACTGTGTGTCCT WF06-R: GGCAACTCAGGTCTGAGGAA	[GACA] ₁₀	45	1.5	246-268	9	0.56	0.521	KC154215
WF12	WF12-F: AGATGGGATGAAAACGTTGG WF12-R: GCCATGATCCTTCCCTTGTA	[ATCT] ₇	45	1.5	256-272	6	0.592	0.662	KC154218
WF16	WF16-F: AAGGGCTGGTTTGGAAAAAT WF16-R: GCTGCAGAGCAACTCACAAA	[AC] ₁₇	54	1.5	213-285	26	0.616	0.803	KC154221
WF18	WF18-F: ATCTGCAGAATAGCCCTTGG WF18-R: GCCGAGTAGGCTCTCTCTCTC	[TC] ₃₃	52	2	100 - 111	11	0.616	0.655	KC154222
WF24	WF24-F: GTGAAGCAGCCGGACATTAT WF24-R: GGGGAGATCCATCCCTACAT	[ATCT] ₂₀	52	2	125-168	11	0.427	0.557	KC154224
WF27	WF27-F: TTCGACACTGCACTTCCTTG WF27-R: TGTGCACAATCGAATGACCT	[GACA] ₁₅	56	1.5	234-394	56	0.803	0.964	KC154226
WF31	WF31-F: AGAGTGC GGAGACATCTGAA WF31-R: TGTCTCGCACACACACACAT	[AG] ₃₅	45	2	258-300	11	0.092	0.161	KC154229
WF32	WF32-F: AATGAGGACAGAGCGGAAAA WF32-R: CGGCAAGTCATGATCAAAAA	[GA] ₉	54	1.5	180-280	20	0.364	0.759	KC154230
WF33	WF33-F: TTCGACACTGCACTTCCTTG WF33-R: TGTGCACAATCGAATGACCT	[GACA] ₁₅	56	1.5	248-376	48	0.69	0.954	KC154231

T_a: annealing temperature

[MgCl₂]: MgCl₂ concentration

bp: fragment size (base pairs)

A: Allelic richness

Ho: observed heterozygosity

He: expected heterozygosity

GB no.: Genbank accession no.

Table 1-2: Characterization of monomorphic microsatellite loci developed from genomic library for winter flounder, *Pseudopleuronectes americanus*.

Locus	primers	repeat	T_a	[MgCl₂]	bp	GB no.
WF03	WF03-F: TTGTGGCAATTCAGAACCAA WF03-R: GGAAGTGTGAGCTTCTCACC	[CTT] ₈	45	2	152	KC154213
WF04	WF04-F: TTGGCGAGCTAATTCAGG WF04-R: GTTGACCCTAATGCGGAAGA	[CTT] ₉	57	2.5	233	KC154214
WF09	WF09-F: ATGGCTGGTGAGGATTTTTG WF09-R: CTGGAATTCGCCCTTGTTA	[GT] ₉	47	2.5	250	KC154216
WF10	WF10-F: GCAGAATTCGCCCTTGTTA WF10-R: GTCGAGAGCGTCGAAGTCAT	[CTT] ₈ [GAA] ₆₀	44	1.5	491	KC154217
WF14	WF14-F: ATATTTGACGTGGGCTTCCA WF14-R: TCATGGTTACGGTTGACGAA	[CTT] ₅	44	1.5	169	KC154219
WF15	WF15-F: TGCAGCCATTAAGTCGATCA WF15-R: GGGTGTAAGTCTCCACGAAATC	[CA] ₂₇	45	2	79	KC154220
WF20	WF20-F TTGGCGAGCTAATTCAGGAT WF20-R: GTTGACCCTAATGCGGAAGA	[CTT] ₇	48	1.5	270	KC154223
WF25	WF25-F: ACGCCGAGAGCCTACAGAG WF25-R: GAGTGTGTGACGCTTTGTGC	[GA] ₃₄	47	2	273	KC154225
WF29	WF29-F: GCTGATGGAGGGTAATTCA WF29-R: GTGGTGCGTGCGTACATAAA	[CA] ₄₀	52	1.5	130	KC154227
WF30	WF30-F: GGGTTTAAGTCTCCAGGAAATCA WF30-R: TTTCTCCGGCGGTTACATTA	[TG] ₃₁	55	2	188	KC154228
WF34	WF34-F: CATGGGTGGACTCTCTCTCAG WF34-R: ATACCCCAAACGGTTTCTC	[TC] ₁₁	46	1.5	411	KC154232
WF35	WF35-F: ACGCCGAGAGCCTACAGAG	[GA] ₃₄	52	1.5	472	KC154233

T_a: annealing temperature

[MgCl₂]: MgCl₂ concentration

bp: fragment size (base pairs)

H_o: observed heterozygosity

H_e: expected heterozygosity

GB no.: Genbank accession no.

Table 1-3: Characterization of 10 species-specific polymorphic loci developed from sub-genomic library for white sharks, *Carcharodon carcharias*.

Locus	Primers	repeat	T_a	[MgCl₂]	H_e	H_o	A	GB no.
CCa83	CCTTAAAAGCACAGAACAAAGATAAA GGGGATTTACAGAGAGCATCC	[TAGA] ₁₆	43	3.5	0.755	0.587	6	KC154203
CCa418*	ACACAGAAGGAGCGTGAAGC TGAGCTGCTTCAGATGGACA	[GA] ₁₁	50	3.5	0.29	0	2	KC154204
CCa711	GATGATTTTGCATGTCACCTTGA CTTTCCTAATATTATTGAGAGAT	[CA] ₁₉	53	3.5	0.81	0.744	11	KC154205
CCa1072	CCCTGTGTTCTTGCACAATG CCATTGAAGCCCTGTGAAGT	[AC] ₂₆	53	3.5	0.795	0.587	12	KC154206
CCa1226*	CTCTGGTTTCCTCCCAAGGT CAGGAGATGGGCACTACACA	[TG] ₁₂	44	2.5	0.729	0.28	9	KC154207
CCa1273*	TGTTTTTGCCTTTTATCCTTGAA CCAGAAACCCACCCAAGTAA	[TG] ₁₀	43	3.5	0.536	0.282	5	KC154208
CCa1276*	CCTAGCATTATGGTCAACATCAG GGTCACTTTCAACTTGAGCAAA	[TG] ₂₁	50	3.5	0.849	0.583	12	KC154209
CCa1419	ATTATCGCATTGGGGGATTT GCAGCAGTCACTCTTGGGTA	[TG] ₁₃	51	3.5	0.708	0.61	6	KC154210
CCa1466	ATGTGTGCAAGCAAGTCTGC GCATAACACCCCCACAGAAG	[TG] ₉ [TG] ₁₁	43	3.5	0.573	0.565	5	KC154211
CCa1536*	ACTCCGGATTGGTGCTATTG TTGGTCCTCCTTTGCTGAAT	[TG] ₁₉	42	3.5	0.761	0.6	13	KC154212

T_a: annealing temperature
[MgCl₂]: MgCl₂ concentration
bp: fragment size (base pairs)
A: Allelic richness
H_o: observed heterozygosity
H_e: expected heterozygosity
GB no.: Genbank accession no.

Chapter 2

Inland incursion of a widespread marine fish leads to highly fragmented population structure and rapidly erodes genetic diversity.

Introduction

Less than 0.01% of aquatic habitats worldwide occur in freshwater, yet these enclosed bodies of water harbor a disproportionate proportion of total fish biodiversity (40%) (Eschmeyer et al. 2010, Horn 1972, Leveque et al. 2008, Lundberg et al. 2000). A number of hypotheses have been advanced to explain this discrepancy, but the leading one is that diversification rates are higher in freshwater habitats because they are more fragmented than marine habitats (May 1994, Stratham 1990, Ward et al. 1994). Marine species have traditionally been thought to exist in relatively large populations connected over large spatial scales by the exchange of larvae and adults, with an absence of substantial vicariant barriers. These characteristics are thought to reduce the probability that populations will become genetically isolated from one another and embark on separate evolutionary trajectories leading to allopatric speciation (Bierne & David 2003, Palumbi 1994, Stratham 1990). In contrast, freshwater fish inhabit relatively fragmented habitats such as rivers and lakes, where populations are limited in size and can become isolated from one another more easily than in the marine environment. Small, inland bodies of water are also frequently vulnerable to rapid environmental changes that cause populations to fluctuate in size, further accelerating genetic differentiation due to genetic drift (i.e., stochastic changes in allele frequencies due to chance) and local selection. Allopatric speciation is considered to be more likely under these conditions (Barraclough et al. 1998, Cracraft 1982, Puebla 2009, Stratham 1990).

Bloom et al. (2013) analyzed diversification rates among the New World silversides (Teleostei, Atherinopsidae), a group of small, surface-dwelling fish comprised of both freshwater and marine species. They concluded that this group originated in the marine environment but has made multiple incursions into freshwater. They found empirical evidence supporting the hypothesis that there are higher diversification rates in freshwater silversides and proposed that this was due to the more fragmented nature of freshwater habitats. Under this model, marine

silversides are proposed to maintain connectivity over broader spatial scales and for longer than freshwater species, thus slowing the rate of diversification. As marine silversides make inland incursions, however, they enter more fragmented habitats and form smaller, more isolated populations that are more prone to genetic drift and local selection, thus encouraging allopatric speciation. While there is macroevolutionary evidence supporting this (Bloom et al. 2013), there are relatively few studies of the population genetics of marine or freshwater silversides to see if the microevolutionary predictions of this hypothesis are met (i.e., that populations of silversides occupying inland bodies of water are smaller, more isolated and their genetic diversity changes more rapidly when compared to silversides occupying the marine environment).

There are three nominal New World silverside species found in southern Florida. *Menidia peninsulae*, the tidewater silverside, is a marine species that is widely distributed in shallow coastal and estuarine habitats of southern Florida and the Gulf of Mexico. In contrast, *Menidia beryllina*, the inland silverside, occurs in freshwater systems in peninsular Florida. The last nominal species is the Key silverside, *Menidia conchorum*. Recent work indicates that this is best described as an ecotype of the more widely distributed tidewater silverside (Conover et al. 2000, Appendix A). An ecotype is a subdivision below the species level adapted to a specific set of environmental conditions found in a distinct habitat and is distinguished by morphological, genetic and physiological differences (Begon 2006, Wilson et al. 1953). Key silversides are endemic to inland, hypersaline pools in the Florida Keys (Conover et al. 2000, Duggins et al. 1986, Getter 1981). Although these are not true freshwater systems, these pools do represent highly fragmented habitats that are not connected to each other and have only very restricted connections to the open ocean if at all. Since the Florida Keys emerged within the last 10,000 years (Hoffmeister & Multer 1968), Key silversides represent a relatively young inland incursion of tidewater silversides, similar to a freshwater incursion from the marine environment. Studying their population genetics offers a rare window into what happens when a marine species enter more fragmented, inland habitats.

Under the Bloom et al. (2013) model of speciation for New World silversides and fundamental population genetic theory (Frankham 1997, 1998), Key silversides are expected to have lower genetic diversity than tidewater silversides. This is due to both the initial founder event(s) coupled with an anticipated cessation of gene flow between populations inhabiting the pools and conspecifics in the ocean and also between populations occupying different pools. The

pools themselves are shallow (typically less than 1 m depth) and are small (mean size = 0.27km², std=0.33 km²), with water levels and salinity fluctuating markedly due to evaporation and precipitation. Each pool population would be expected to be relatively small, subject to marked changes in size and therefore prone to genetic drift and inbreeding, two processes that would further reduce intrapopulation diversity and promote genetic differentiation. Here, we test the Bloom et al. (2013) hypothesis for increased rate of speciation among silversides occupying inland habitats on a microevolutionary scale. We measured genetic structure and changes in genetic diversity among Key silversides inhabiting seven pools in the Florida Keys over a time period of 14 generations (1999 and 2012). We also compare their genetic diversity to that of two marine populations of their parent species, the tidewater silverside, separated from each other by ~ 100 km.

Methods

Sample acquisition

Sample locations in the Gulf of Mexico and the Florida Keys, along with abbreviations used for sample locations throughout this paper are shown in Figure 2-1. We recovered archived specimen collected by Conover et al. (2000) from February 20 – 26, 1999. These were initially collected in from 18 different hypersaline pools, in the Florida Keys using a 30 m small-mesh beach seine. A handheld Global Positioning System (GPS) was used to record the location of each pool. Whole fish from each pool were preserved in alcohol and later dried and stored in plastic bags in a chest freezer. From March 8 – 10, 2012 we revisited 14 sites where Key silversides had previously been found (Conover et al. 2000). Salinity and temperature was measured and depth estimated at each location. A 30 m beach small-mesh beach seine was used to sample fish from each pool (Florida Fish and Wildlife Conservation Commission Permit # SAL-12-1366-SRP). Key silversides were identified, held on ice and then frozen prior to genetic analysis. Tidewater silversides were collected using a 21 m center-bag seine that was hauled along mangrove shorelines and on offshore flats in Charlotte Harbor, Florida, a flood plain estuary that opens to the Gulf of Mexico (26 49.236 -82 15.335) and Sarasota Bay (27 29.465 - 82 40.083).

Laboratory procedures

Genomic DNA was extracted from 0.015 – 0.035g of tissue using the Qiagen Blood and Tissue extraction kit (Qiagen, Valencia, CA, USA). Nine previously published microsatellite loci

(Sbrocco 2011) were amplified in a 10 μ l reaction containing genomic DNA, 1xPCR buffer, 10x bovine serum albumin, 1.5 – 3.5mM MgCl₂, 0.12 mM dNTPs, 0.16 μ M of the reverse primer and fluorescently labeled m13 primer, 0.04 μ M of the species specific forward primer and 1 U Taq polymerase. Thermal cycling profiles consisted of 4 minutes at 94°C followed by 30 cycles of 94°C for 15 seconds, primer specific annealing Temperature T_a for 15 seconds and 72°C for 45 seconds, concluding with 5 cycles of 94°C for 15 seconds, 53°C for 15 seconds and 45 seconds at 72°C with a final extension at 72°C for 10 minutes. The amplified products were separated into single strands and run on a Genetic Analyzer (Applied Biosystems ABI31370 sequencer) with an internal size standard (LIZ-500 Applied Biosystems). A subset of 8 homozygotes for each locus was re-amplified at 3°C below the primer-specific T_a to determine if reduced stringency would result in the amplification of null alleles (i.e. we would expect additional alleles to be amplified for individuals previously scored as homozygotes). Alleles were scored by single analyst (SO) using Peakscanv1.0 (Applied Biosystems). A subset of approximately 30% of genotypes was verified by a second analyst (KAF).

Data analysis

Spatial and temporal differentiation between and within Key silverside sites were determined by estimating global and pairwise F_{ST} and testing for significance as implemented in FSTAT (Goudet 1995). In addition, we used STRUCTURE (Pritchard et al. 2000) a Bayesian clustering approach to determine spatial and temporal clusters of individuals. The clusters must be genetically different (F_{ST} > 0.05, Latch et al. 2006) in order to be detected. STRUCTURE was run using the admixture and non-admixture model using a priori information regarding the sampling location. Spatial structure was analyzed separately for pools in 1999 and in 2012. We simulated K = 1 – 10 for 5 independent runs each to determine convergence for a burn-in period of 15,000 Markov chain Monte Carlo (MCMC) steps followed by 350,000 MCMC steps.

The genetic diversity for each sample site for all silversides was characterized by calculating the expected and observed heterozygosity, H_e and H_o (Nei 1978) and the allelic richness as implemented in FSTAT (Goudet 1995). Exact tests implemented in GENEPOP (Raymond & Rousset 1995, Rousset et al. 2008) were used to test for linkage disequilibrium and deviations from Hardy-Weinberg Equilibrium (HWE). To determine the effect of loci out of HWE on the statistics calculated, each parameter was were calculated both including and excluding loci out of HWE. F_{IS} was calculated using FSTAT (Goudet 1995) to measure

heterozygote deficiency resulting from inbreeding at the sample level. In addition, we used STORM (Frasier 2008) to calculate the internal relatedness (IR) which measures the level of relatedness of the parents of an individual (Amos et al. 2001). Outbred individuals will have an $IR \leq 0$ while individuals derived from consanguineous mating would have a positive value, with 1 being the maximum value.

N_e was estimated for each sample location and sampling time (i.e., 2012 and 1999) using the linkage disequilibrium implemented in LDNE (Waples & 2006, 2008) with a lowest included allele frequency $p_{crit} = 0.03$. This method estimates N_e based on the small amount of linkage of alleles occurring due to sampling error during recombination and only requires a single temporal sample. Three tests were used to detect genetic bottlenecks: the M-ratio test (Garza & Williamson 2001) which tests for recent bottlenecks, the mode shift test (Luikart et al. 1998), and heterozygote excess test which are implemented in BOTTLENECK (Piry et al. 1999). The tests are based on the assumption that when a population bottleneck occurs, rare alleles will be lost first.

Results

Eighteen of 29 pools sampled in 1999 contained Key silversides (Conover et al. 2000). Fifteen of these pools were relocated in 2012 while 3 locations (Key West, Crocodile Lakes and Highway Creek) were no longer accessible and were not re-sampled. Key silversides were recovered from the remaining pools, with sample numbers ranging from 2 to over 100; an average 44 individuals were caught per haul. Salinity ranged from 37.2 – 46ppt with the exception of Key Largo, which had the lowest salinity at 33.1 ppt. For the analysis of genetic diversity we selected seven pools for which we had a sufficient sample size from both 1999 and 2012 to estimate genetic diversity (Hale et al. 2012). Seven microsatellite loci were amplified in 469 Key silversides from seven pools. For comparisons of genetic diversity 108 tidewater silversides collected from Charlotte Harbor and Sarasota bay were used.

Among the seven pools containing Key silversides pairwise F_{ST} for all comparisons between pools sampled in the same year (i.e., 1999 or 2012) were highly significant ($p < 0.001$) and remained significant after Bonferroni adjustment for multiple comparisons (Table 2-1). Temporal pairwise F_{ST} calculated between individuals sampled in the same pool between 1999 and 2012 were also highly significant ($p < 0.001$) and remained significant after Bonferroni adjustment for multiple comparisons (Table 2-1C). We used the ΔK metric suggested by Evanno et al. (2005) to determine the statistically most supported number of clusters. For the analysis of

structure between the pools in 1999, $K=3$ was indicated to be the most likely number of clusters. There was a second peak detected for $K=6$. For the pools sampled in 2012 the strongest peak was detected for $K=5$, with secondary peaks at $K=3$ and $K=7$ (Figure 2-2). Bayesian inference of genetic structure is depicted in Figure 2-3 showing the individual membership coefficients for each cluster. Overall, all measures of population differentiation (F_{ST} and Bayesian clustering) concur that each pool forms a distinct population. In addition, temporal analysis indicates strong shifts allele frequency shifts within each pool from 1999 to 2012 (13 generations).

The heterozygosity, allelic richness and inbreeding coefficient F_{IS} of each Key silverside pool sample from 1999 are presented in Table 2-2, and the genetic diversity measure of Key silversides and tidewater silversides sampled in 2012 are presented in Table 2-3. There was no evidence of linkage disequilibrium between any pair of loci (data not shown). Deviations from Hardy-Weinberg Equilibrium (HWE) due to heterozygote deficiency were detected at approximately 50% of all locus, sample location and sample time combinations (112 combinations). For multi-locus comparisons all sample locations except one Key silverside population in one year (BTK in 1999) and the Charlotte Harbor tidewater silversides were out of HWE. In general, the genetic diversity of each Key silverside population as described by its heterozygosity ($H_o= 0.26-0.667$) and allelic richness ($A=5.29-10.29$) dropped between the 1999 and 2012 samples with the exception of CjK (Figure 2-4).

The inbreeding coefficient F_{IS} calculated over all loci was positive and significantly different from zero for all sample locations and sample periods, ranging from 0.233-0.514 with the exception of BTK in 1999 (0.041) and CK sampled in 2012 (0.166). Again, the general pattern was an increase of F_{IS} indicating an increase in inbreeding between 1999 and 2012 with the exception of CK and KL. Evidence of inbreeding was further corroborated through high average IR values for sample pools (Figure 2-5). With the exception of CK and CjK levels of internal relatedness increased between 1999 and 2012. During both sample periods levels of internal relatedness were high, ranging from 0.1291 – 0.5441 ($SD \pm 0.24-0.26$) in 1999 and 0.3104 – 0.6623 ($SD \pm 0.26-0.30$) in 2012. Overall the mean IR was 0.464, which indicates that on average the parents of an individual are related at the level of full siblings.

Estimates of N_e (Table 2-3) for each Key silverside population dropped significantly from 1999 to 2012. Again, CK was the exception where N_e increased from 253.9 to 645.2. In 1999, the confidence intervals were also much wider with the upper confidence limit being

infinity in each case. In 2012, with the exception of CK, all pools had definitive C.I. limits. M-ratios were calculated for each sample location for both sample periods using the global number of alleles r found in all pools (Table 2-4). The M-ratios were very low with mean values over all loci ranging from 0.26 – 0.41, indicative of a recent or ongoing population bottlenecks. There was no distinct pattern of systematic increasing or decreasing M-ratios between the sample years. No evidence for bottlenecks were detected using mode shift test and heterozygosity excess tests ($p=0.3 - 0.6$).

Discussion

The analysis of the spatial structure shows that the Key silverside is not panmictic across the Florida Keys, which is in line with the highly fragmented nature their habitat. Gene flow between populations occupying different hypersaline pools is generally absent or negligible. Both F_{ST} and Bayesian clustering confirmed there were marked differences in allele frequencies between pools in both 1999 and 2012. Although some of the pools are only a short distance apart and located on the same Key, it does not appear that adult fish or their eggs are transported between pools (e.g., during floods or hurricanes) frequently enough to have any homogenizing effect. The one exception was Cudjoe Key where a local resident informed us that after the pool had partially dried out during the summer, on more than one occasion, fishermen (who use the silversides for bait) have added silversides from other pools in the past. In contrast, we found only modest differentiation between tidewater silversides sampled ~ 100 km apart in Sarasota Bay and Charlotte Harbor, two estuaries connected to the open ocean. Pairwise F_{ST} between these two marine sites were an order of magnitude smaller than values between inland pool populations of Key silversides separated by as little as 2 km. Although there are relatively few other population genetic studies of New World silversides, there is evidence of long-range dispersal and genetic homogeneity over large spatial scales in Atlantic silversides (*Menidia menidia*) along the U.S. eastern seaboard (Conover 1998).

The existence of Key silversides in these hypersaline pools has been documented since at least the 1920s (Getter 1981, Hildebrand & Ginsburg 1927), representing nearly 100 generations for this short-lived, rapidly maturing species. In the absence of gene flow between populations in different pools genetic differences are expected to arise based on stochastic changes in allele frequencies due to genetic drift, which is inversely proportional to the effective population size. Point estimates for N_e were low in all pools in both 1999 and 2012. There was also a distinct

drop in N_e in six of seven pools between 1999 and 2012. Notably, the upper confidence was infinity in all pools in 1999 but < 100 in six of them by 2012, further indicating that the effective population size of each pool decreased in the 13 years (13 generations) between sampling events. The effective population size of Sarasota Bay and Charlotte Harbor tidewater silversides were larger in 2012 than all of the pools frequently by 1-2 orders of magnitude and the latter had an unbound upper confidence interval. The effective population sizes of Key silversides are comparable to those found in highly structured freshwater fish populations, including sticklebacks ($N_e=15-39$, Araguas 2012, $N_e=12-86$, Seymour 2013), brown trout ($N_e=16-32$, Sanz 2011), $N_e=63$, Charlier 2011), Tokyo bitterling ($N_e=5-28$, Kubota 2010) and purple spotted Gudgeon ($N_e=30-60$, Hughes 2012).

The estimated effective population sizes of Key silversides in each of the pools we examined are at levels where they would be expected to be highly susceptible to genetic drift (Frankham 1996, Lynch & Lande 1998). Together with selection, genetic drift is believed to be one of the critical microevolutionary forces promoting allopatric speciation (Coyne 1992, Lande 1980). We observed low N_e at the two locations tidewater samples are estimated, though it is difficult to determine how large N_e really is, as we did not sample enough different locations along the coast to determine population structure. When estimating N_e for a combined sample, N_e appears to be sufficiently large to avoid loss of genetic diversity. In all seven pools we observed substantial changes in genetic diversity between 1999 and 2012. These changes were large enough that F_{ST} and Bayesian clustering analysis indicated that each pool population was significantly differentiated between the two sampling periods. In six of them, allelic richness and/or observed heterozygosity decreased while measures of inbreeding at the population and individual level increased. In CK all of these parameters increased, although this was likely due anthropogenic introduction of new fish. We cannot determine whether the reduction in genetic diversity observed in the other six pools occurred gradually over the entire 14 years or during specific discrete bottleneck events, such as a particularly dry year causing the pool size and silverside population to experience an unusually large decline. Nevertheless, either scenario is consistent with the Bloom et al. (2013) hypothesis that isolated populations of silversides occupying inland habitats are prone to rapid genetic change that could contribute to reproductive isolation and eventual allopatric speciation. We show that these changes in genetic diversity are possible over as little as 13 years in silversides, most likely due to their short life cycles.

It is possible that most or all of the genetic changes we observed in Key silversides over the 13-year period were due to selection as opposed to genetic drift, or a combination of them. Microsatellite loci themselves are anticipated to be selectively neutral but can be acted upon by selection if they are closely linked to a coding region (Jarne & Lagoda 1996, Wiehe 1998). It is unknown whether or not this is the case for the loci used in this study but it is unlikely that all seven loci would be linked to coding regions. All of the patterns we observed were common across all or most of the loci, which is more suggestive of random drift acting on all of them than selection operating on a subset of them. Nevertheless it is possible that selection is driving the success of a small number of lineages in these pools, thereby reducing genetic diversity and N_e over time. Future work could look at changes in coding regions or apply comparative genomics approaches to disentangle the relative roles of drift and selection in shaping the genetic changes between and within pool populations.

The inland incursion(s) of tidewater silversides into the Keys has produced a complex of disconnected populations that have small N_e . We show large reductions in all metrics of genetic diversity occurring in six of seven populations over a 13-year period, as well as an increase in inbreeding and reduction in N_e over the same time period. Our results argue against Key silversides forming one cohesive species in the future. Instead, the configuration of isolated, rapidly changing gene pools is more likely to form a species flock if populations in multiple pools survive for a long enough period to develop reproductive isolation from their parent species and Key silversides in other pools. Although genetic drift, selection or bottlenecks occurring between the two sampling periods could all alone or in combination explain the declines in genetic diversity, they are all consistent with the hypothesis that fish that living in fragmented inland habitats have small, rapidly changing gene pools, which underpins their more rapid diversification when compared to marine fish (Benton 2001, Betancur 2010, Bloom et al. 2013, Eschmeyer et al. 2010, May 1994, Stratham 1990).

Figure 2-1: Sample locations for *M. conchorum* and *M. peninsulae* in the Florida Keys and the Gulf of Mexico, respectively. Location numbers correspond to Conover et al. 2000 report. Abbreviations in parenthesis are used throughout text, figures and tables.

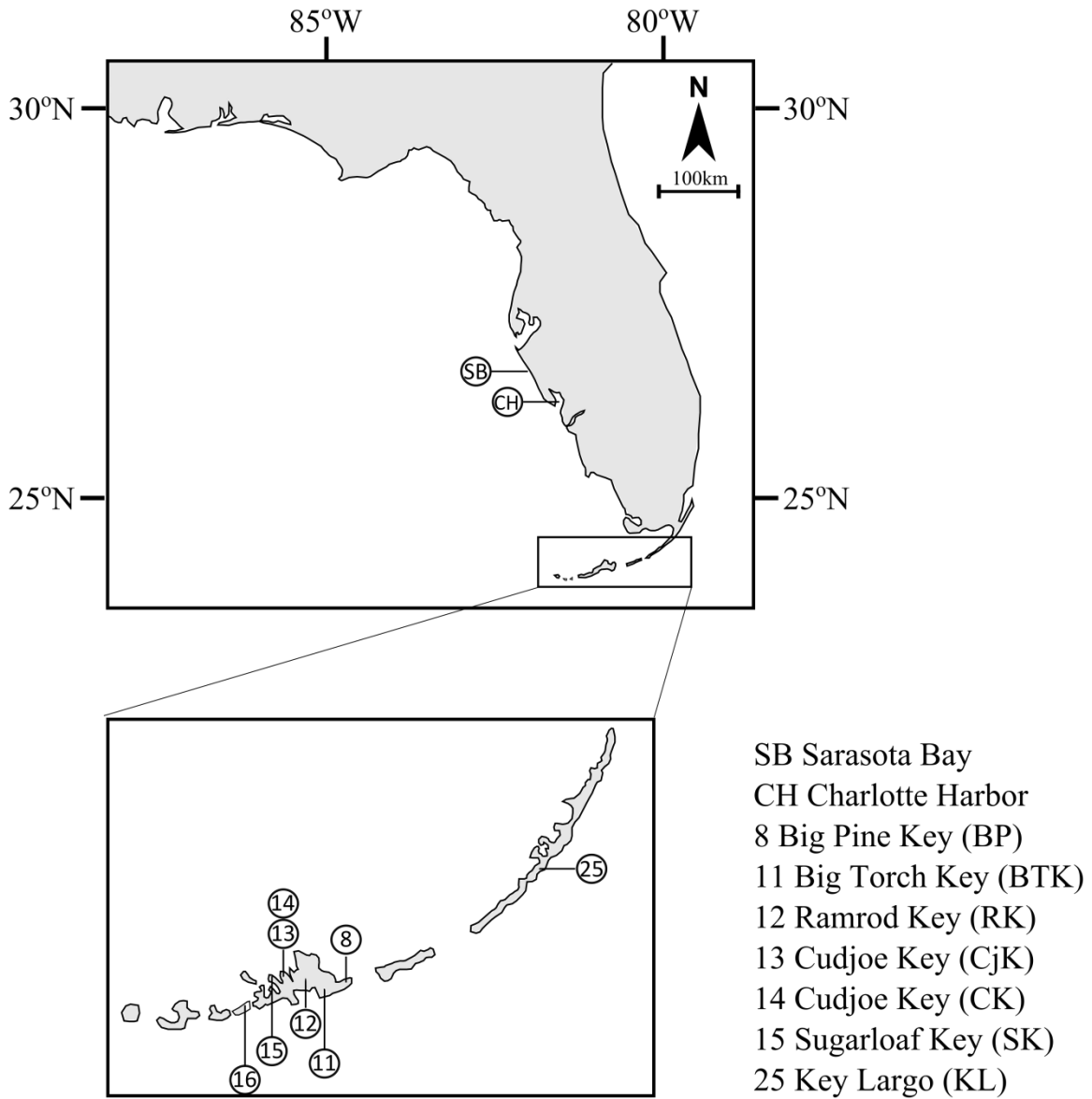


Figure 2-2: Evanno et al (2005) method to determine the number of clusters of individuals K for 1999 and 2012. (A) convergence of mean $L(K) \pm SD$ over 5 runs for $K = 2 - 10$ using no admixture model with prior location information. (B) $\Delta K = \text{mean}(L''(K))/SD(L(K))$. The sharp peak indicates highest level of structure and the most likely value for K .

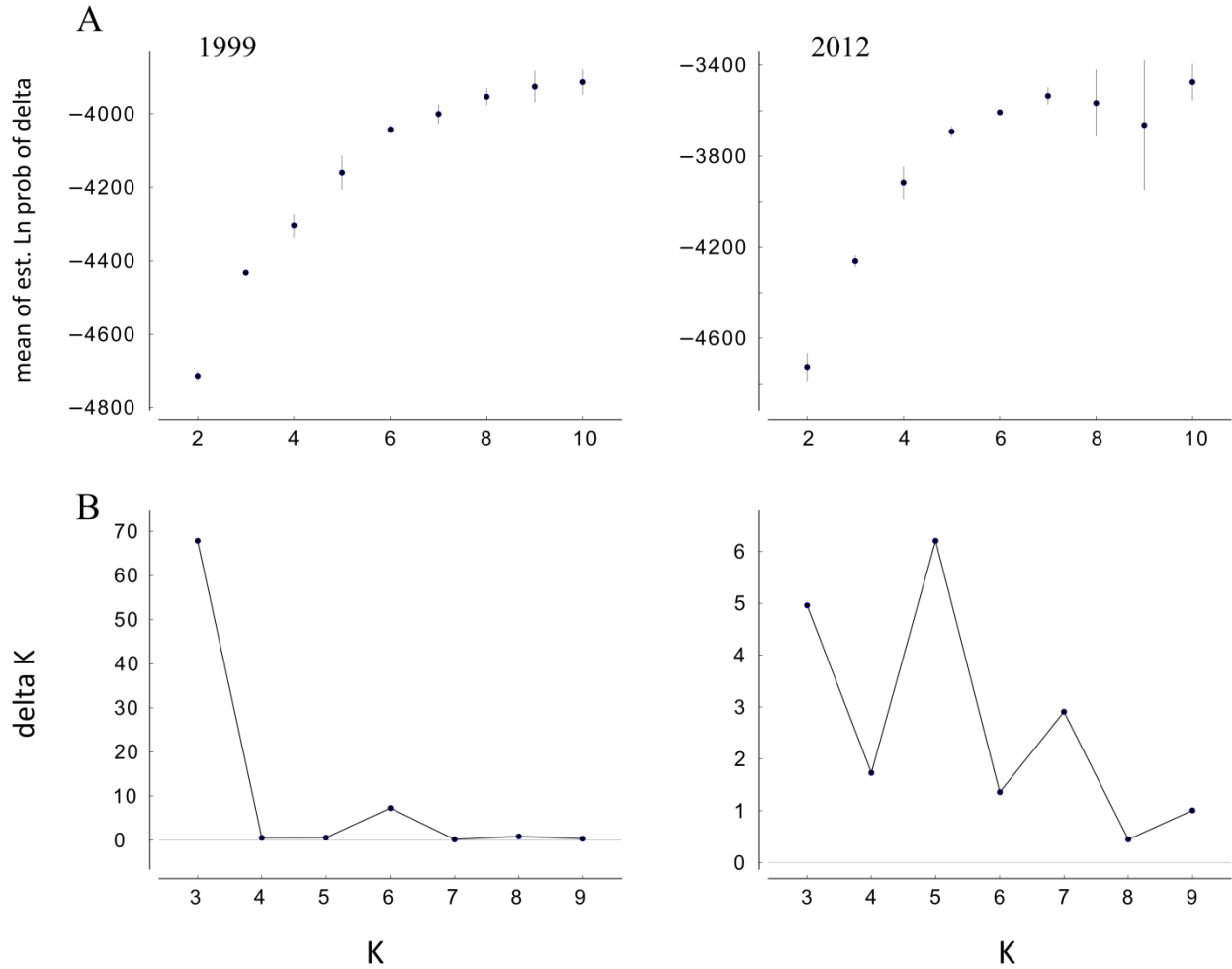
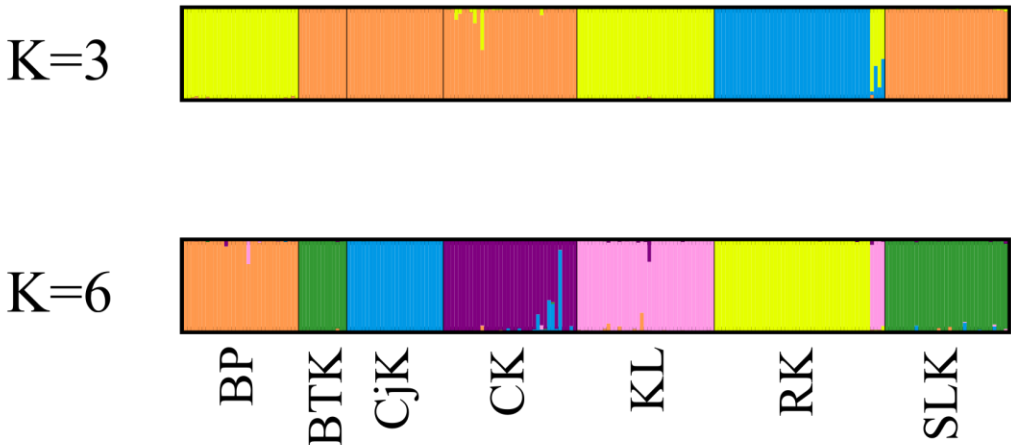


Figure 2-3: Bayesian inference of genetic structure. STRUCTURE results for no admixture model for K=2-17 and sample locations (on x-axis) as a priori information. Individual membership coefficients for each cluster are represented by a single vertical line. (A) Individual membership coefficients for 1999. (B) Individual membership coefficients for 2012.

A



B

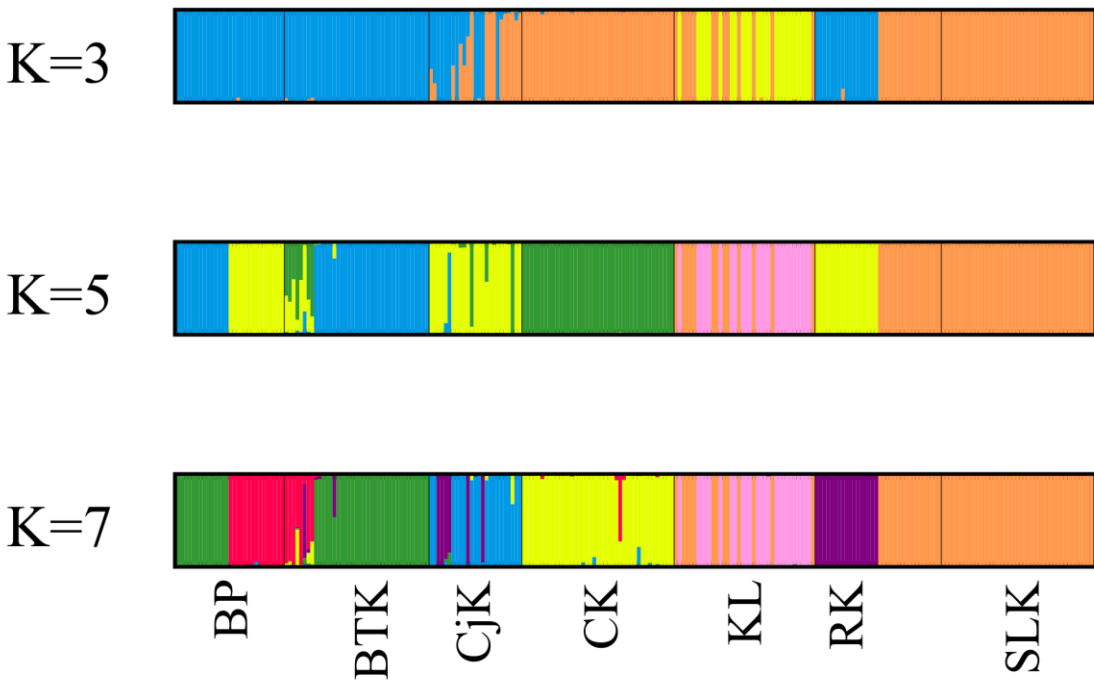


Figure 2-4: Changes in genetic diversity between 1999 and 2012. Multi-locus allelic richness and heterozygosity indicated for 1999 (circles) and 2012 (squares). Trajectories are indicated by arrows.

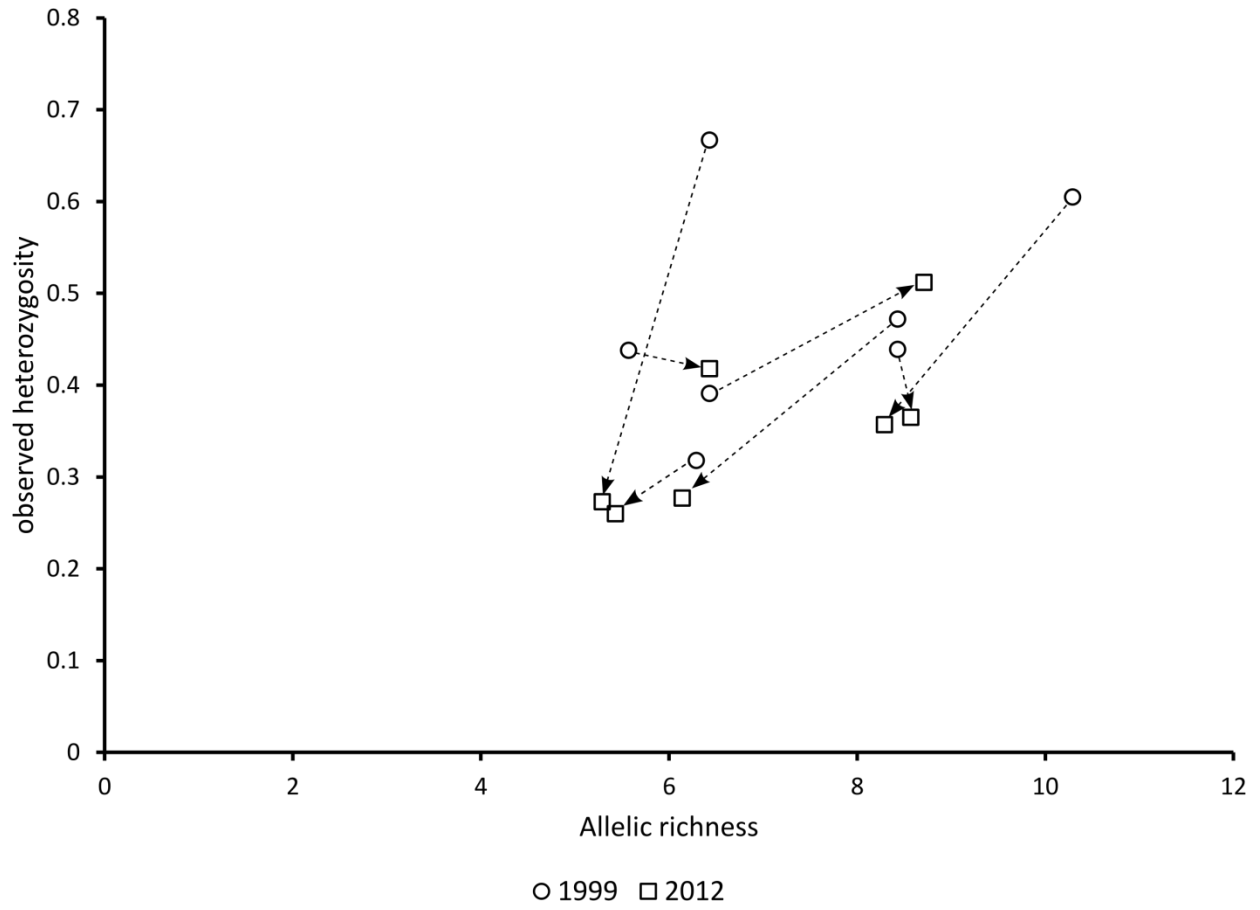


Figure 2-5: Internal Relatedness (IR) for all *M. conchorum* sample locations in 1999 and 2012. Trajectories of average IR-values for each sample location indicated by arrows.

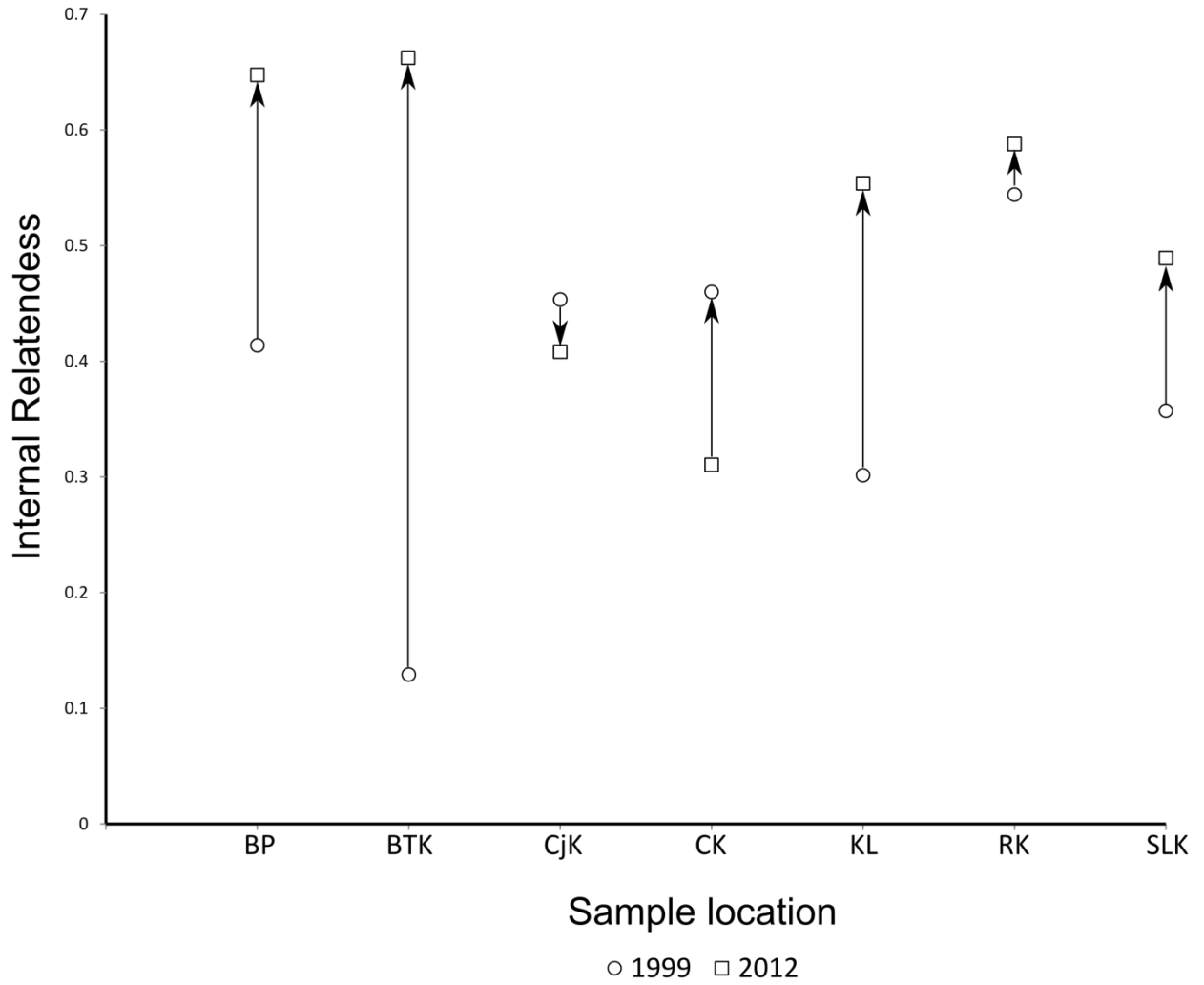


Table 2-1: Temporal and spatial analysis of population structure using pairwise F_{ST} . (A) Pairwise F_{ST} of all seven pools sampled in 1999. (B) Pairwise F_{ST} of all pools re-sampled in 2012. (C) Temporal comparison for each pool. All values were significant to a level of $p < 0.001$ and remained significant after Bonferroni correction for multiple comparisons ($p < 0.05$).

A.

	BTK	CjK	CK	KL	RK	SLK
BP	0.1528	0.1658	0.1348	0.0872	0.3269	0.1312
BTK	0	0.1512	0.1595	0.1320	0.2635	0.1103
CjK		0	0.1431	0.1556	0.2985	0.105
CK			0	0.1242	0.2532	0.0886
KL				0	0.2345	0.1290
RK					0	0.2632

B.

	BTK	CjK	CK	KL	RK	SLK
BP	0.1372	0.1213	0.2093	0.2537	0.2099	0.3272
BTK	0	0.3178	0.3262	0.3812	0.3947	0.4488
CjK		0	0.1365	0.1785	0.1535	0.2336
CK			0	0.168	0.1948	0.1836
KL				0	0.1966	0.198
RK					0	0.182

C.

location	FST
BP	0.2804
BTK	0.3561
CjK	0.1561
CK	0.1135
KL	0.0532
RK	0.2748
SLK	0.1211

Table 2-2: Genetic diversity for all sample pools of *M. conchorum* in 1999. Genetic diversity is described by individual and multi-locus heterozygosity, allelic richness and inbreeding coefficients for all sample pools for *M. conchorum* in 1999. Sample locations and loci with significant deviations from Hardy-Weinberg Equilibrium are indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$). No linkage disequilibrium was detected for any sample locations at any location.

<i>All loci</i>	BP	BTK	CjK	CK	KL	RK	SLK
<i>N</i>	31	13	26	36	37	46	33
<i>H_o</i>	0.472	0.667	0.438	0.391	0.605	0.318	0.439
<i>H_e</i>	0.684	0.696	0.587	0.593	0.804	0.567	0.571
<i>A</i>	8.43	6.43	5.57	6.43	10.29	6.29	8.43
<i>F_{IS}</i>	0.314	0.041	0.257	0.345	0.525	0.448	0.233
Mm272	***		***	***	***		*
<i>H_o</i>	0.367	0.846	0.04	0.156	0.361	0	0.788
<i>H_e</i>	0.802	0.714	0.713	0.771	0.759	0	0.76
<i>A</i>	10	6	6	8	11	1	9
<i>F_{IS}</i>	0.547	-0.195	0.945	0.8	0.528	NA	-0.037
Mm248				*	***	***	*
<i>H_o</i>	0.065	0.385	0	0.171	0.459	0.522	0.03
<i>H_e</i>	0.063	0.495	0	0.255	0.7	0.733	0.088
<i>A</i>	2	3	1	4	12	13	2
<i>F_{IS}</i>	-0.017	0.231	NA	0.332	0.347	0.29	0.66
Mm108	*	**		*	***	***	
<i>H_o</i>	0.462	0.3	0.667	0.25	0.679	0	0.583
<i>H_e</i>	0.649	0.679	0.802	0.589	0.836	0.758	0.585
<i>A</i>	5	5	6	3	9	5	7
<i>F_{IS}</i>	0.292	0.571	0.172	0.583	0.191	1	0.003
Mm251	***				***	**	***
<i>H_o</i>	0.467	0.556	0.5	0.75	0.556	0.615	0.458
<i>H_e</i>	0.843	0.627	0.556	0.544	0.785	0.785	0.933
<i>A</i>	10	6	6	4	8	7	18
<i>F_{IS}</i>	0.451	0.121	0.102	-0.394	0.315	0.176	0.514
Mm204	***		*	***		*	*
<i>H_o</i>	0.69	0.909	0.769	0.5	0.778	0.543	0.719
<i>H_e</i>	0.822	0.926	0.845	0.763	0.867	0.643	0.831
<i>A</i>	14	12	9	9	12	6	13
<i>F_{IS}</i>	0.164	0.02	0.091	0.347	0.105	0.157	0.137
Mm240				***	***	***	***
<i>H_o</i>	0.733	0.923	0.778	0.639	0.735	0.297	0.5
<i>H_e</i>	0.778	0.822	0.837	0.892	0.925	0.733	0.797
<i>A</i>	9	10	7	14	17	8	9
<i>F_{IS}</i>	0.058	-0.129	0.072	0.287	0.207	0.598	0.376
Mm02	***		*			*	
<i>H_o</i>	0.524	0.75	0.318	0.267	0.667	0.25	0
<i>H_e</i>	0.83	0.607	0.358	0.337	0.733	0.362	0
<i>A</i>	9	3	4	3	3	4	1
<i>F_{IS}</i>	0.375	-0.286	0.114	0.211	0.111	0.313	NA

N: sample size

H_o: observed heterozygosity

H_e: expected heterozygosity

A: Allelic richness (Number of alleles/per locus)

F_{IS}: inbreeding coefficient

Table 2-3: Genetic diversity for all sample pools of *M. conchorum* and two *M. peninsulae* sample locations in the Gulf of Mexico in 2012. Loci with significant deviations from Hardy-Weinberg Equilibrium are indicated by *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$). No linkage disequilibrium was detected for any sample locations at any location.

<i>All loci</i>	BP	BTK	CjK	CK	KL	RK	SLK	CH	SB
<i>N</i>	29	39	25	41	38	34	41	33	34
<i>H_o</i>	0.277	0.273	0.418	0.512	0.357	0.260	0.365	0.483	0.610
<i>H_e</i>	0.565	0.429	0.589	0.612	0.662	0.493	0.489	0.624	0.724
<i>A</i>	6.14	5.29	6.43	8.71	8.29	5.43	8.57	10.00	11.29
<i>F_{is}</i>	0.514	0.368	0.295	0.166	0.465	0.468	0.258	0.181	0.178
<i>Mm272</i>			***		***	**	***	*	
<i>H_o</i>	0.240	0.026	0.160	0.537	0.278	0.063	0.345	0.581	0.962
<i>H_e</i>	0.372	0.026	0.643	0.581	0.785	0.560	0.739	0.621	0.762
<i>A</i>	2	2	7	9	10	5	11	10	12
<i>F_{is}</i>	0.360	0.000	0.755	0.077	0.649	0.890	0.538	0.067	-0.063
<i>Mm248</i>	*	*							
<i>H_o</i>	0.320	0.360	0.417	0.049	0.147	0.375	0.000	0.233	0.406
<i>H_e</i>	0.566	0.641	0.451	0.048	0.165	0.476	0.000	0.303	0.472
<i>A</i>	4	4	3	3	3	5	1	6	6
<i>F_{is}</i>	0.439	0.443	0.078	-0.006	0.113	0.215	NA	0.095	0.053
<i>Mm108</i>	***	***	**	***	*	***			
<i>H_o</i>	0.208	0.462	0.278	0.600	0.459	0.077	0.293	0.690	0.719
<i>H_e</i>	0.808	0.838	0.675	0.609	0.723	0.554	0.287	0.777	0.800
<i>A</i>	7	8	6	7	6	5	6	9	9
<i>F_{is}</i>	0.746	0.454	0.595	0.015	0.368	0.863	-0.021	0.082	0.103
<i>Mm251</i>	*		***	*	***		***	***	***
<i>H_o</i>	0.407	0.000	0.727	0.455	0.289	0.063	0.488	0.606	0.750
<i>H_e</i>	0.679	0.000	0.860	0.814	0.785	0.063	0.863	0.920	0.948
<i>A</i>	8	1	12	15	12	2	22	19	21
<i>F_{is}</i>	0.404	NA	0.158	0.445	0.634	0.000	0.438	0.258	0.125
<i>Mm204</i>	*				***	***	***	**	*
<i>H_o</i>	0.138	0.000	0.400	0.711	0.211	0.324	0.659	0.455	0.500
<i>H_e</i>	0.574	0.000	0.402	0.836	0.701	0.683	0.802	0.702	0.673
<i>A</i>	5	1	4	10	13	9	9	10	13
<i>F_{is}</i>	0.763	NA	0.006	0.152	0.703	0.530	0.180	0.186	0.092
<i>Mm240</i>	**	***	**	***	***			***	***
<i>H_o</i>	0.556	0.471	0.652	0.538	0.474	0.750	0.415	0.667	0.522
<i>H_e</i>	0.885	0.836	0.835	0.813	0.770	0.789	0.413	0.848	0.878
<i>A</i>	14	9	11	11	6	7	6	13	10
<i>F_{is}</i>	0.376	0.445	0.223	0.340	0.388	0.050	-0.004	0.173	0.333
<i>Mm02</i>						***			
<i>H_o</i>	0.074	0.595	0.294	0.296	0.640	0.200	0.353	0.152	0.412
<i>H_e</i>	0.073	0.662	0.258	0.586	0.709	0.329	0.321	0.195	0.532
<i>A</i>	3	12	2	6	8	5	5	3	8
<i>F_{is}</i>	-0.010	0.103	-0.143	-0.191	0.100	0.397	-0.103	0.142	0.080

N: sample size

H_o: observed heterozygosity

H_e: expected heterozygosity

A: Allelic richness (Number of alleles/per locus)

F_{is}: inbreeding coefficient

Table 2-4: Estimates of effective population size (N_e). N_e estimates using the linkage disequilibrium method are shown for each sample location in 1999, 2012 and over both sample periods. [95% Confidence interval] estimated using jackknifing method.

Sample location	1999	2012	overall
BP	180.8 [33.7, ∞]	7.6 [4.4, 12.0]	11.1 [8.9, 13.6]
BTK	90.6 [77.5, ∞]	10.4 [2.8, 74.1]	29.5 [26.5, 32.7]
CjK	87.4 [54.6, ∞]	16.9 [6.7, 112.0]	31.9 [21.1, 53.7]
CK	253.9 [51.7, ∞]	645.2 [84.8, ∞]	145.1 [70.3, 1381]
KL	72.5 [64.8, ∞]	16.5 [12.3, 22.5]	47.9 [35.8, 67.8]
RK	135.1 [1.4, ∞]	5.1 [2.9, 8.1]	25.4 [18.8, 35.6]
SLK	47.7 [17.2, ∞]	12.8 [2.8, 125.7]	17.5 [4.5, 78.7]
CH		108.5 [46.6, ∞]	
SB		43.7 [28.9, 79.2]	
all <i>M. peninsulae</i>		4620.0 [143.9, ∞]	

Table 2-5: M-ratios for all locations (1999/2012) for each individual locus and multi-locus mean value.

	BP	BTK	CjK	CK	KL	RK	SLK
Mm272	0.56/0.11	0.28/0.11	0.33/0.39	0.44/0.50	0.65/0.64	0.06/0.28	0.50/0.61
Mm248	0.11/0.21	0.16/0.21	0.05/0.16	0.26/0.18	0.69/0.18	0.65/0.26	0.11/0.05
Mm108	0.26/0.37	0.26/0.26	0.32/0.32	0.16/0.37	0.47/0.32	0.26/0.26	0.37/0.32
Mm251	0.34/0.28	0.21/0.03	0.21/0.41	0.21/0.52	0.29/0.42	0.24/0.07	0.62/0.78
Mm204	0.74/0.26	0.63/0.05	0.47/0.21	0.49/0.53	0.65/0.70	0.32/0.47	0.68/0.47
Mm240	0.33/0.52	0.37/0.33	0.26/0.41	0.56/0.43	0.68/0.22	0.31/0.26	0.33/0.22
Mm02	0.02/0.03	0.02/0.02	0.01/0.02	0.03/0.02	0.03/0.01	0.02/0.01	0.02/0.01
mean	0.40/0.04	0.29/0.26	0.26/0.28	0.33/0.40	0.51/0.41	0.29/0.27	0.38/0.39

Chapter 3

Severe inbreeding and small effective number of breeders in a formerly abundant marine fish

Introduction

McNeely et al. (1990) defined three levels of biodiversity: ecosystem diversity, species diversity and genetic diversity. While ecosystem diversity describes the differences of habitats and environmental parameters that shape communities, species diversity describes the variety and abundance of organisms inhabiting a certain area and genetic diversity focuses on the combination and variation of genes found within a single population of one species. The conservation of genetic diversity is often not well incorporated into marine fish management (Kenchington & Heino 2002) despite being the most fundamental level of biodiversity and a Key source of variation that enables evolutionary adaptation (Frankham et al. 1999, Willi et al. 2006). This stems from the fact that the Key processes that reduce genetic diversity, such as inbreeding and stochastic genetic drift, are associated with very small, fragmented populations and are assumed to be diluted in large, well-mixed populations (Allendorf 1986, Frankham 1996, Lacy 1987). Since marine fish are traditionally assumed to exist as large, panmictic populations connected by larval and adult-mediated dispersal (Cowen & Sponaugle 2009, Frisk et al. 2013, Smedbol et al. 2002) it is not surprising that conservation of genetic diversity is not emphasized in marine fish conservation (Kenchington & Heino 2002, Ward et al. 1994).

Recent studies have shown that marine fish populations can be more structured than traditionally thought (Poulsen et al. 2006, Thorrold et al. 2001, Wang et al. 2007) and effective population size N_e , which determines how vulnerable populations are to losing genetic diversity due to genetic drift, can be up to five orders of magnitude smaller than census population size (N_c) in broadcast spawning species that exhibit large variance in reproductive success (Hauser et al. 2002, Hoarau et al. 2005, Poulsen et al. 2006). These findings have initiated a paradigm shift that marine fish may be more vulnerable to processes that reduce genetic diversity than previously assumed (Hoarau et al. 2005, Turner et al. 2002), for example through inbreeding, defined in population genetics as a departure from random mating. Hoarau et al. (2005) detected

heterozygote deficiencies in plaice (*Pleuronectes platessa*) in the North Sea and, after ruling out alternative hypotheses, concluded that inbreeding was responsible for this pattern. Despite having a relatively large N_c , plaice tend to spawn in their natal area and have high variance in reproductive success, increasing the probability that spawning pairs or groups will contain related individuals. Despite this remarkable finding, there have been few follow-up studies of inbreeding in marine fish, even though heterozygote deficiencies have been detected in many other species, including redfin culter (*Culter erythropterus*) (Wang et al. 2007), anchovy (*Engraulis encrasicolus*, Zarraonaindia et al. 2009), rockfish (*Sebastes melanops*, Lotterhos et al. 2012) and whitefish (*Coregonus lavaretus*, McCairns et al. 2012). It is therefore difficult to determine whether inbreeding in plaice is an anomaly or a process that should be of broader conservation concern for heavily exploited marine fish.

The winter flounder (*Pseudopleuronectes americanus*) is a demersal flatfish that was once very common in the inshore bays and estuaries of the Northwest Atlantic and supported very large commercial and recreational fisheries (Buckley 1989, Hendrickson et al. 2006). The species' geographic range encompasses the North American coast from Labrador to Georgia (McCracken 1963). Spawning migrations occur in the winter and spring months and there are four broadly defined and genetically discrete spawning stocks across the species range: Sable Island Bank, St. Mary's Bank, Browns Bank, and Georges Bank (McClelland et al. 2005). Winter flounder eggs are demersal and it has been assumed that pre-settlement larvae are mixed through larval dispersal within each stock (Chant et al. 2000). However, more recent studies have shown that larvae are likely retained within their natal estuary (Buckley et al. 2008) and a number of authors believe that adults remain within or return to their natal estuaries to spawn (Lobell 1939, Perlmutter 1947, Sagarese & Frisk 2011, van Guelpen & Davis 1979, Wuenschel et al. 2009), both of which could promote the development of fine-scale population structure.

Winter flounder began declining in the late 1980s and the age structure of many populations has become truncated, with a low proportion of the remaining fish older than 5 years (Hendrickson et al. 2006). Long Island, New York (LI) is a very densely populated region with 2,086 people per km², and has a long history of commercial and recreational exploitation of winter flounder (Hendrickson et al. 2006). Commercial (NMFS 2007, Northeast Fisheries Science Center 2011) and recreational (Northeast Fisheries Science Center 2011, <http://www.st.nmfs.noaa.gov/recreational-fisheries/access-data/run-a-data-query/index>) landings

have reached record low levels, and despite management efforts there is little evidence of recovery (Buckley et al. 1991, Buckley et al. 2008, DeLong et al. 2001, Hendrickson et al. 2006). In light of the extent of inbreeding observed in North Sea plaice, a fish with many life-history similarities to winter flounder, we tested for inbreeding in winter flounder in LI estuaries. We also tested for genetic differentiation among bays, estimated the effective number of breeders (N_b) for each bay and tested for genetic bottlenecks, all of which contribute to the rate at which population genetic diversity is lost.

Methods

Sample collection

Young-of-the-year (YOY) winter flounder were collected with a 1m beam trawl every two weeks from June to October in 2010 and May to October in 2011. Samples were collected in 6 bays (Figure 3-1): Jamaica Bay (40 38' 28.43" N, 73 49' 02.37" W) in 2010 and 2011, Moriches (40 47' 02.47"N, 72 47' 23.14"W) in 2010 and 2011, Hempstead (40 36' 58.85"N, 73 35' 52.81" W) in 2011, Shinnecock Bay (40 51' 46.13"N, 72 29' 44.73"W) in 2010 and 2011, Cold Spring Pond (40 53' 59.04"N, 72 27' 40.31"W) in 2010, and Napeague Harbor (41 00' 34.62"N, 72 02' 49.84"W) in 2010. With the exception of Hempstead Bays, trawls within each bay occurred at 10 randomly chosen stations within a grid along a section of coast where winter flounder had previously been caught. Supplementary sampling occurred in 2011 throughout Hempstead Bay using a beam trawl or 3-30m beach seines due to low abundances of fish in this area. Fin clips were taken from all flounder and stored in 75% reagent grade ethanol at room temperature.

Laboratory analysis

Genomic DNA was extracted from fin clips (0.015 – 0.035g of tissue) using the Qiagen Blood and Tissue extraction kit (Qiagen, Valencia, CA, USA). Eleven microsatellite loci were amplified using PCR primers that were either used for previous winter flounder microsatellite studies (five loci, Crivello et al. 2004, McClelland et al. 2005) or recently developed (six loci, O'Leary 2013a). The master mix consisted of 1x PCR buffer, 10x bovine serum albumin, 1.5 – 3.5 mM MgCl₂, 0.12 mM of each dNTPs, 0.16 μM of the reverse primer and the fluorescently labeled M13 primer and 0.04 μM of the species specific forward primer and 1 unit Taq polymerase in a final reaction volume of 10 μl. Thermal cycling profiles were 4 minutes at 94°C, 30 cycles of 94°C for 15 seconds, primer specific annealing temperature for 15 seconds, 72°C for

45 seconds and 5 cycles of 94°C, 15 seconds at 53°C and 45 seconds at 72°C with a final extension for 10 minutes at 72°C. Locus-specific annealing temperatures (T_a) are given in Table 3-1. Amplified products were separated and sized using an internal size standard (LIZ-500 Applied Biosystems) on a Genetic Analyzer (Applied Biosystems ABI3730 sequencer). Alleles were scored by a single analyst (SO) using Peakscanv1.0 (Applied Biosystems). For quality control, a second analyst (KAF) verified the scoring of approximately 30% of heterozygotes and 100% of the homozygotes.

Testing for technical artifacts

The entire genotypic database was analyzed with MICROSATELLITE TOOLKIT FOR EXCEL (Park 2001) and MICROCHECKER (van Oosterhout et al. 2004) to check for possible scoring errors, identical genotypes, large allelic dropouts, null alleles and large allelic gaps. Null alleles occur when there is a mutation within the binding site of the primer, causing annealing failure during PCR. If null alleles exist in the population, some individuals will be homozygous for these alleles at a certain locus and will consistently fail to amplify at this locus despite working at others. If null alleles were occurring at high frequencies we would therefore expect chronic issues with gaps within the dataset. To assess this possibility we attempted to re-amplify any samples that failed on the first attempt, making a dedicated effort to obtain a genotype at every locus for all individuals. When null alleles are present a proportion of individuals scored as homozygotes are actually heterozygotes for a null allele. We attempted to reveal these “false” homozygotes by reducing the stringency of the PCR. Following Hoarau et al. (2005) a subset of eight homozygotes for each locus was re-amplified at lower temperatures (3° C lower than the T_a listed for each locus in Table 3-1) to promote the amplification of null alleles, i.e. if null alleles were present we would expect additional alleles to be amplified in an individual previously scored as homozygotes thus revealing a proportion of homozygotes to be false.

Statistical analysis

Expected and observed heterozygosity (Nei 1978) and allelic richness were calculated for each sampled estuary using FSTAT (Goudet 1995). GENEPOP (Raymond & Rousset 1995, Raymond et al. 1997) was used to test for linkage disequilibrium and deviations from Hardy-Weinberg Equilibrium (HWE) using exact tests. Global F_{ST} was calculated to estimate population differentiation and pairwise F_{ST} to assess genetic differentiation between bays and tested for significance as implemented in FSTAT (Goudet 1995). The shortest distance by sea

between each pair of sample locations was measured using Google Earth V.6.2.2.6613 to assess isolation by distance (IBD). The relationship between genetic similarity ($M = (1/F_{ST}) - 1$)/4 and geographic distance was evaluated using Mantel tests (Mantel 1967, Slatkin 1995) as executed in IBDWS (Jensen et al. 2005).

Deviations from HWE can arise from inadvertent grouping of multiple populations into one or from analyzing a large number of related individuals (Wahlund effect). To test for the possibility of genetically distinct groups of winter flounder spawning in the same bay at different times we calculated pairwise F_{ST} for several temporal groups. I first tested all samples caught in 2010 (N=115) against those caught in 2011 (N=99) and then pooled samples caught in the early sampling season (May – July) and the late sampling season (August – October) and calculated pairwise F_{ST} for the following four temporal groups: early 2010 (N=89), late 2010 (N=26), early 2011 (N=70) and late 2011 (N=29). The program STRUCTURE (Pritchard et al. 2000) was also used to estimate the number of distinct genetic populations in LI by using a Bayesian approach to detect clusters of individuals that would minimize disruptions from HWE within the whole sample set. STRUCTURE was run using the admixture and the non-admixture model both using and not using a priori information regarding the sampling location. We simulated $K = 2-15$ for 10 independent runs each to determine convergence with a burn-in period of 15,000 Markov chain Monte Carlo (MCMC) steps followed by 350,000 MCMC steps. This approach is capable of detecting if there were strongly differentiated groups ($F_{ST} > 0.05$, Latch et al. 2006) spawning in the same bays at different times that were admixed in the collected sample. Lastly, the pairwise relatedness of all individuals was tested and the average within group relatedness of individuals calculated at each sample location to detect family structure within each sample.

The effective number of breeders (N_b) was estimated for each bay and LI using the linkage disequilibrium method as implemented in LDNE (Waples & Do 2008) with the lowest included allele frequency $p_{crit} = 0.02$. This method estimates N_b based on a single sample by using the small level of linkage of alleles that occurs due to sampling error during recombination. Since we used young-of-the-year individuals sampled over 1-2 years, the parameter we estimated is N_b that effectively produced the sampled cohorts, not N_e for the whole generation (Robinson et al. 2013). Three tests for genetic bottlenecks were implemented for LI as a whole and for each bay: the M-ratio test (Garza & Williamson 2001), the mode shift test (Luikart et al. 1998) and the

heterozygote excess test. The last two methods are implemented in the program BOTTLENECK (Piry et al. 1999).

Three metrics were used to estimate levels of inbreeding. At the sample (i.e., bay) level, the inbreeding coefficient F_{IS} (Wright 1984) was used to measure the intrapopulation heterozygosity deficiency resulting from inbreeding when alleles are shared by descent. At the individual fish level, the internal relatedness (IR) was used to measure the relatedness of individual's parents (Amos et al. 2001). For outbred individuals IR should be close to or below zero, whereas individuals derived from consanguineous mating have an IR that is positive (to a maximum of 1), indicating that the parents of that individual shared many alleles and are closely related. The program STORM (Frasier 2008) was used to calculate IR for each individual. Pairwise t-tests were used to test for significant differences between the mean IR levels of each sample time and location. In addition the homozygosity levels of each individual was calculated using STORM (Frasier 2008), which indicates the proportion of loci within the genotype of an individual that are homozygous.

Results

Eleven microsatellite loci were amplified in 267 individuals sampled in 6 LI bays (32–66 individuals per bay; Figure 3-1). Loci had from 6 to 56 alleles (mean = 25, s.d. = 15.8) and observed heterozygosities ranging from 0.364 to 0.764 (Table 3-1). MICROCHECKER did not detect evidence of stuttering or large allelic drop out at any locus. While MICROCHECKER did suggest the presence of null alleles due to strong deviations from HWE, none of the homozygotes exhibited an additional allele after being re-amplified at lower annealing temperatures (N=8 homozygotes per locus). All individuals were successfully amplified and genotyped at all loci, indicating that there were no individuals that were homozygous for null alleles (i.e., there were no blanks in the dataset).

The global F_{ST} was small but significantly different from zero ($F_{ST} = 0.008$; $p < 0.05$). Pairwise F_{ST} values between bays were consistently significant ($p < 0.05$) for Napeague Harbor (except compared to Hempstead Bay) and Shinnecock Bay (Table 3-2), though they were no longer significant after Bonferroni correction for multiple comparisons. Analysis of IBD (Figure 3-2) was also significant, though weak ($r^2 = 0.0958$, $p < 0.01$). Even after removing Napeague Harbor, the most divergent and distant site, the IBD pattern was still significant ($r^2 = 0.0701$, $p < 0.01$). Temporal pairwise F_{ST} calculated between the years 2010 and 2011 was 0.005 ($p = 0.4$);

pairwise F_{ST} -values for comparison between the early and late sampling season ranged between 0.005 and 0.010 ($p = 0.3 - 0.6$). Bayesian clustering implemented in STRUCTURE failed to detect population structure for the number of clusters K ranging from 2 to 15 for the entire sample set. Additionally, analysis of pairwise relatedness of all sampled individuals showed that less than 6% were related at a level of half-siblings or higher ($r > 0.25$).

Overall genetic diversity as measured by heterozygosity and allelic richness was similar among all bays (Table 3-3). The estimated effective number of breeders for the sampled bays and LI overall were low (Table 3-4) ranging from 65 to 262 breeding individuals per bay and 966 overall. The L-mode shift test exhibited a non-bottlenecked distribution of alleles and the heterozygote excess test for bottlenecks was not significant ($p < 0.98$). The M-ratios for the LI collection as a whole were generally high, whereas all loci but three (A441, J42 and WF12) exhibited moderate to low M-ratios within individual bays when using the entire range (R) of alleles found in all LI locations (Table 3-5). Large, significant discrepancies between expected and observed heterozygosities were detected at all loci in all bays (Table 3-1). Global Hardy-Weinberg testing of heterozygote deficiency were statistically significant at all bays ($p < 0.001$ for all sample locations) and all loci ($p = 0.00 - 0.0043$ except WF06, $p = 0.7$). F_{IS} values over all loci were significantly different from zero and positive in all six bays, ranging from 0.169 – 0.283 (Figure 3-3). All loci except WF6 exhibited this pattern. The average in-group relatedness r per bay was $r = -0.052 - 0.004$. 94% of all values of pairwise relatedness were $r < 0.2$. Average IR of individual fish was high, though highly variable (mean 0.229, s.d. 0.206) with a range of IR from -0.178 – 0.999 (Figure 3-4). This pattern was common to all 6 bays (Figure 3-4). In addition, pairwise comparison of mean IR of sample location shows some significant differences between sample locations (Table 3-6). Moriches and Napeague have significantly higher mean IR values than Cold Spring Pond, Hempstead and Jamaica Bay ($p < 0.05$), while Shinnecock has a significantly higher mean IR value than Cold Spring Pond. The internal level of homozygosity (HL), i.e. the proportion of loci of an individuals' genotype that were homozygous, ranged from $HL = 0 - 1$. The mean value of the internal homozygosity level of individuals as highest for Moriches and Napeague with $HL = 0.414$ (std=0.172) and $HL = 0.427$ (std=0.172), respectively, and lowest for Cold Spring Pond and Hempstead Bays ($HL = 0.296$, std=0.168 and $HL = 0.314$, std=0.166) indicating that on average 30-40% of an individuals loci are homozygous.

Discussion

Young of the year winter flounder living in New York estuaries exhibit relatively high genetic diversity in terms of microsatellite allelic richness, yet the low M-ratios observed suggest that rare alleles may have been lost within individual sample locations. Genetic diversity was weakly geographically partitioned between some of the bays, with distance between sites playing a small but significant role in driving this structure. All bays were out of HWE due to large excesses of homozygotes across 10 of 11 loci, leading to high inbreeding coefficients (F_{IS}) in all bays. Many individuals also exhibited very high internal relatedness and individuals' genotypes exhibited a high proportion of homozygous loci. These patterns could not be explained by an artificial inflation of homozygosity resulting from technical issues. Large allelic dropout produces a pattern skewed towards homozygotes for small alleles, which did not occur in the LI winter flounder. If null alleles were at high enough frequencies at all loci to drive these patterns, I should have observed many null homozygotes (blanks for certain loci) when there were none. I would probably have also been able to amplify null alleles in homozygotes by lowering annealing temperatures, but this did not occur. Inadvertent sampling of closely related individuals can also generate HWE deviations of this nature, but few (~ 6%) of the sampled young-of-the-year fish exhibited high relatedness ($r > 0.25$) ruling out a Wahlund effect due to sampling of closely related individuals. A Wahlund effect due to undetected population structure is also unlikely, as no cryptic genetic structure within any of the bays was detected using STRUCTURE.

The most likely explanation for these large deviations in HWE is that inbreeding is occurring in LI winter flounder. Similar evidence for inbreeding has been documented in a wide variety of terrestrial and freshwater animals (e.g. wolves (*Canis lupus*, Liberg et al. 2005), deer (*Cervus elaphus*, Slate et al. 2000), wasps (*Ancistrocerus antilope*, Chapman & Stewart 1996), brook trout (*Salvelinus fontinalis*, Castric et al. 2002), but relatively few marine fish (Hoarau et al. 2005, Langen et al. 2011). A number of other studies have found that marine fish populations can exhibit strong deviations from HWE (e.g., European anchovy (*Engraulis encrasicolus*, (Zarraonaindia et al. 2009), whitefish (*Coregonus lavaretus lavaretus*, McCairns et al. 2012), rockfish (*Sebastes melanops*, Gomez-Uchida & Banks 2006), including other flatfish and winter flounder in other regions (Anderson & Karel 2012, Crivello et al. 2004, Dongdong et al. 2012, Florin & Hoglund 2008, Hemmer-Hansen et al. 2007, McClelland et al. 2005). In these studies excess homozygosity is generally attributed to technical or sampling artifacts and alternative

biological explanations, such as inbreeding, are not explicitly tested. Our results and those of Hoarau et al. (2005) suggest that inbreeding should be routinely considered as a potential cause of HWE deviation in heavily exploited marine fish. Loci with the largest deviations from equilibrium expectations are frequently discarded in studies of wild animal populations based on the assumption that they have null alleles (Dhamarajan et al. 2012). While this is always a possibility, it is important to consider that biological explanations are also an option, especially if the deviations are chronic at multiple loci. This will enable rigorous testing of these alternative explanations and a less biased assessment of the magnitude and causes of HWE deviation in marine fish.

I propose that the inbreeding observed in LI populations may be due to a confluence of a small spawning population in each bay and a propensity of these fish to spawn in their natal estuary. It has generally been assumed that marine fish exhibit panmictic population structure and do not require management at the sub-population level (Smedbol et al. 2002); however studies have shown that population structure is important in many species (Frisk et al. 2013). Previous research on LI proposes the existence of multiple distinct behavioral groups with observations indicating the presence of resident and migratory individuals termed "bay fish" and "offshore fish", respectively (Lobell 1939, Poole 1966, Sagarese & Frisk 2011). Poole (1966) estimated morphometric differences and variation in age and growth across four south shore bays of LI. It is not yet clear if these contingents are genetically differentiated (Frisk et al. 2013, Sagarese & Frisk 2011), but if they are, then including individuals from both groups in the same analysis could cause heterozygote deficiencies relative to HWE expectations. However, this hypothesis is unlikely to explain the strong HWE deviations observed. Bayesian clustering failed to detect any strongly differentiated groups that could correspond to "bay" and "offshore" contingents within bays and none of the young-of-the-year fish from different cohorts in the same year sampled in a given bay were genetically differentiated from one another. Although I report small but significant F_{ST} along the south shore of LI and Peconic Bays and a weak pattern of IBD, these estimates of genetic differentiation are confounded by inbreeding at the individual level within the sampled bays. Since there were significant differences in IR between bays it cannot be assumed that this bias is the same for each bay. More direct methods for assessing migration rates between bays (tagging, telemetry or otolith microchemistry) are needed to assess genetic differentiation between winter flounder in these bays.

I suggest that relatively few mature adults are contributing to each generation, resulting in a high proportion of related fish spawning with each other. Like any broadcast spawning species it is probable that there is large variation in reproductive success in this species due to high larval and pre-recruit mortality (DeLong et al. 2001). When spawning populations are large, inbreeding is unlikely even despite these characteristics. All recent indicators, however, show that spawning populations have reached extremely low levels in New York estuaries (Sagarese & Frisk 2011, Socrates 2006, Yencho 2009). There is no direct evidence that spawning fish are related because sampled individuals were YOY rather than spawning adults. However, our estimates of N_b producing the sampled cohorts were consistent with the premise that there are relatively few spawning adults in these bays, because all estimates were in the tens to hundreds of individuals. It is important to note that these estimates do not necessarily reflect the spawning population for the entire bay. They may only be representative of the part of each bay that was sampled since bays were not sampled randomly and flounder are patchily distributed (Sagarese & Frisk 2011).

Inbreeding could directly contribute to the failure of some marine fish to recover from exploitation as it has been linked to lower survival and reproduction rates and lower resistance to disease and environmental stress (Keller & Waller 2002) and can have a significant effect on extinction risk (O'Grady et al. 2006), with persistence time of inbred endangered species being reduced 17.5 – 28.5% (Liao & Reed 2009). Additionally, the effect of inbreeding depression is multiplied in a stressful environment (Armbruster & Reed 2005). A recent study by Bickley et al. (2012) tested the effect of an endocrine disrupter (the fungicide clotrimazole) on reproduction on a model fish (zebrafish, *Danio rerio*) in a laboratory setting. They confirmed that inbreeding has a much stronger effect when combined with exposure to a chemical stressor, resulting in lower embryo viability and few offspring. Western populations of winter flounder on LI, particularly those in Jamaica Bay, are exposed to anthropogenic habitat degradation, particularly large amounts of municipal sewage effluent which has been shown to contain estrogenic compounds (Ferguson et al. 2001, Todorov et al. 2001). Additionally there is evidence that winter flounder from Jamaica Bay demonstrate signs of endocrine disruption linked to the estrogenic compounds found there (Mena et al. 2006). This environmental degradation of LI bays combined with inbreeding depression may contribute to the ongoing recruitment failure of winter flounder and should be further investigated.

Marine fish have historically been managed without an underlying concern that populations could be reduced to the point where they would become vulnerable to local extinction and processes that reduce their genetic diversity. The effective number of breeders is shown to be so low in some parts of the winter flounder range that it is able to be estimated using genetic approaches that work most effectively for small populations. Although only weak evidence of a genetic bottleneck is observed at this stage, the number of spawning adults is sufficiently low for inbreeding to be occurring. Given similar findings in genetic studies of North Sea plaice (Hoarau et al. 2005) and evidence of local population structure in other species, I suggest that resource managers should consider the possibility that exploited marine fish are vulnerable to local extinction and inbreeding.

Figure 3-1: Sample locations for winter flounder (2010-2011). Sample locations are indicated by black circles, number of individuals sampled (N) in parenthesis.

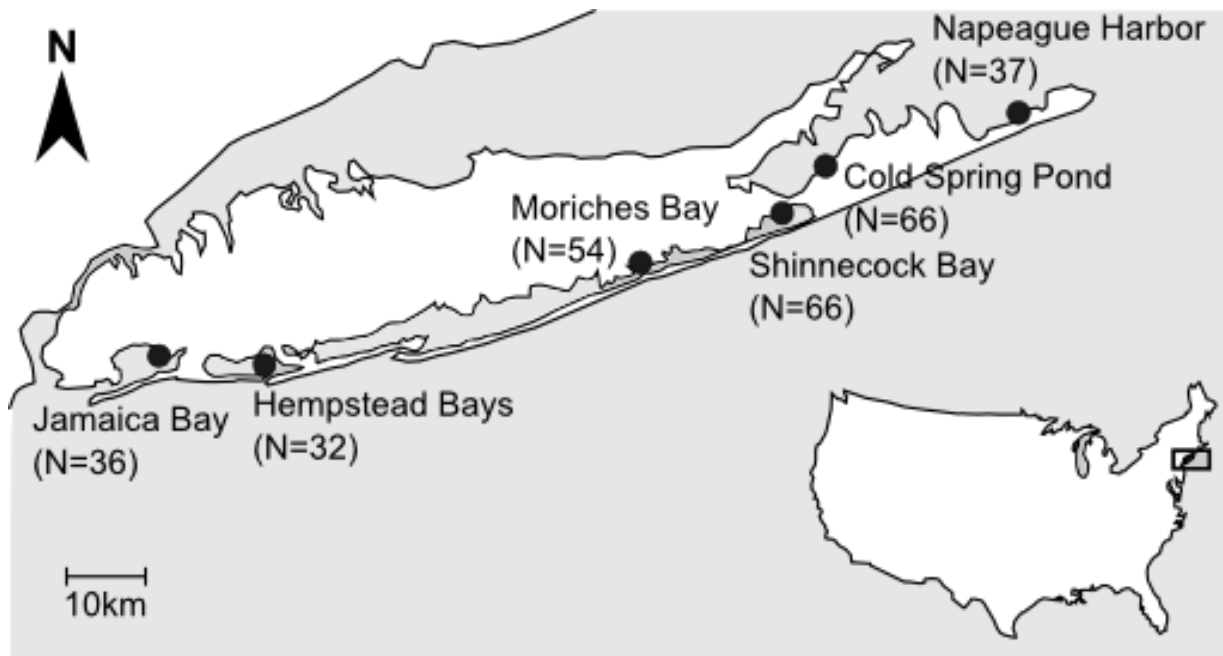


Figure 3-2: Isolation by distance. Regression of pairwise geographic distance and genetic similarity. (A) All sample populations. (B) All sample locations excluding Napeague.

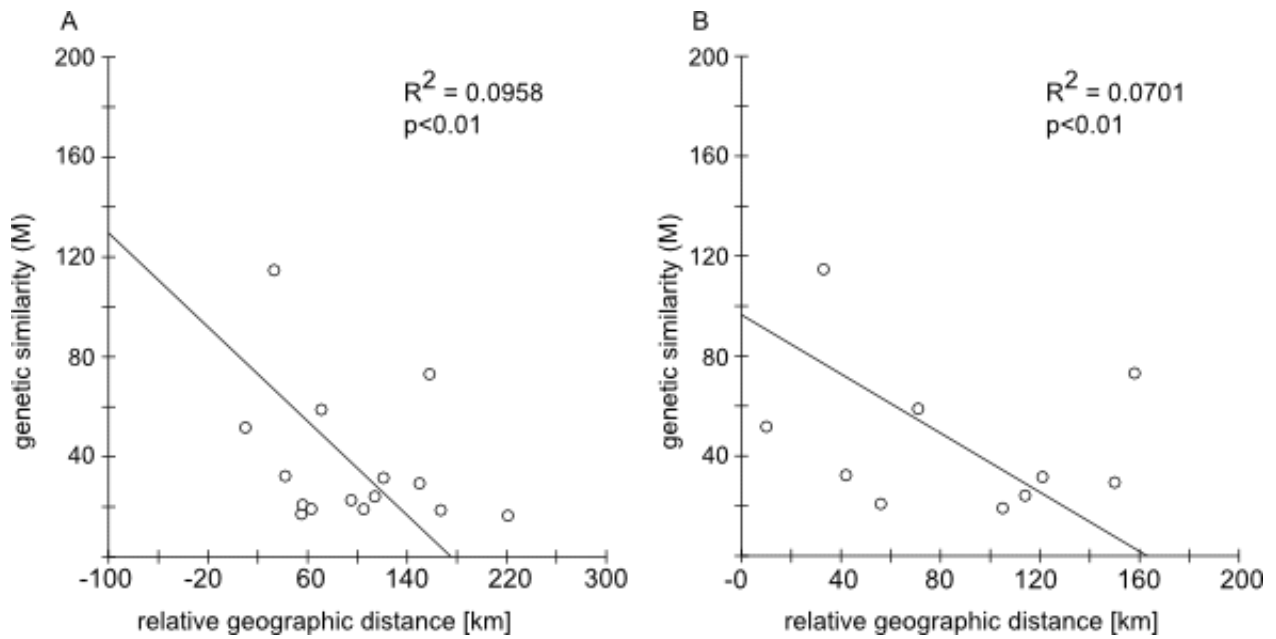


Figure 3-3: Inbreeding coefficient F_{IS} . Inbreeding coefficient for each locus for each sample location.

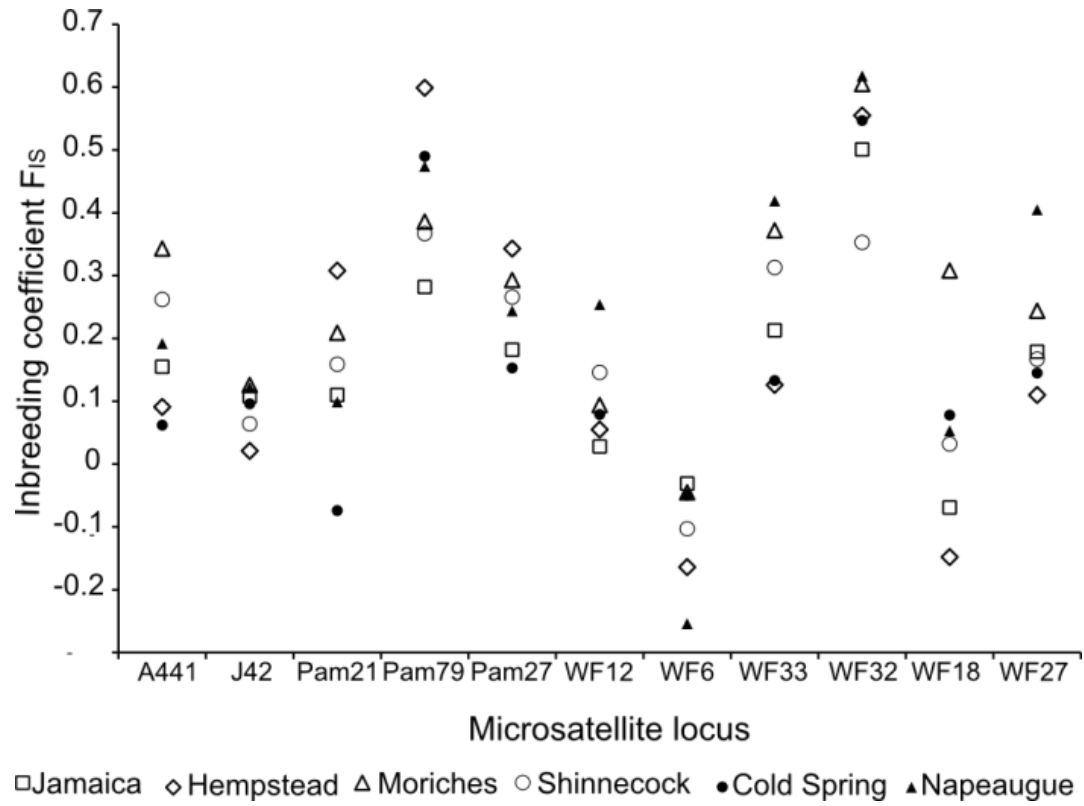


Figure 3- 4: Internal relatedness. Frequency distribution of level of internal relatedness (IR) over all loci for all sample locations.

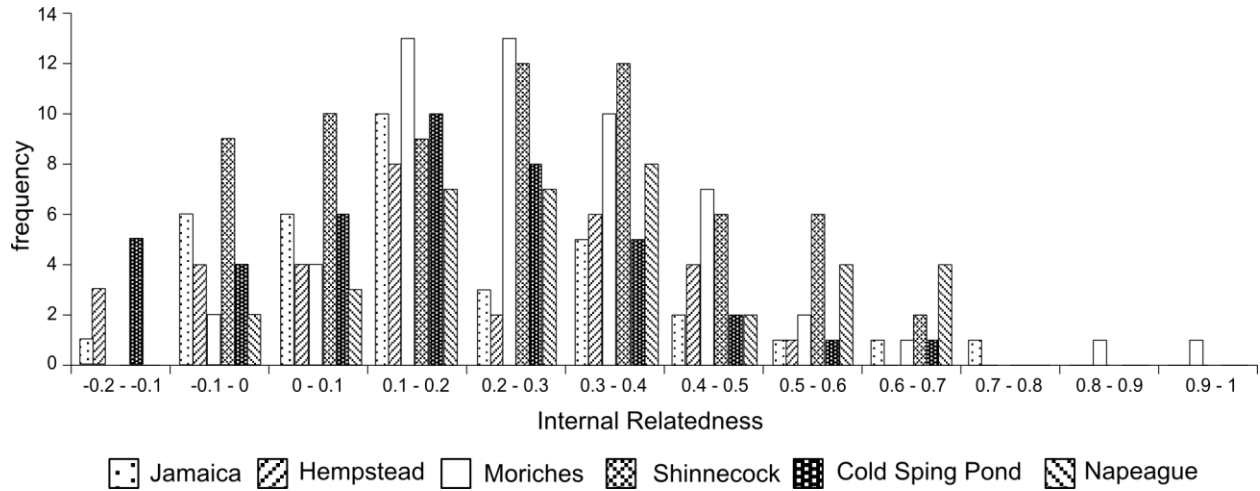


Table 3-1: Genetic diversity of winter flounder in Long Island bays. Genetic diversity is described for each microsatellite locus over all sample locations through allelic richness and heterozygosity.

Locus	T_a	K	H_o	H_e
A441	52	17	0.688	0.862
J42	52	19	0.764	0.844
Pam21	46	16	0.677	0.787
Pam27	47	20	0.638	0.869
Pam79	43	38	0.564	0.956
WF06	45	9	0.561	0.520
WF12	45	6	0.591	0.662
WF16	54	26	0.616	0.803
WF27	56	56	0.758	0.963
WF32	54	20	0.364	0.758
WF33	56	48	0.689	0.954

T_a : Annealing temperatures

K: number of alleles

H_e: expected heterozygosity

H_o : observed heterozygosity

Table 3-2: Pairwise F_{ST} for all sample locations. F_{ST} values are given above the diagonal and p-values are presented below the. Significant values before Bonferroni correction are indicated. Indicative adjusted nominal level (5%) for multiple comparisons is: 0.0033.

	Cold Spring	Jamaica	Hempstead	Moriches	Napeague	Shinnecock
Cold Spring		0.0104	0.004*	0.008	0.014**	0.005
Jamaica	0.187		0.012	0.005	0.013	0.013*
Hempstead	0.023	0.120		0.008	0.015*	0.009*
Moriches	0.093	0.280	0.150		0.011**	0.002**
Napeague	0.003	0.060	0.023	0.010		0.013**
Shinnecock	0.387	0.027	0.037	0.010	0.007	

* ($p < 0.05$)

** ($p < 0.01$)

Table 3-3: Multi-locus genetic diversity for each sampling location (sample size N) and overall Long Island.

Location	N	H_e	H_o	A
Cold Spring	42	0.8255	0.701	16.18
Hempstead	32	0.8264	0.656	14.09
Jamaica	36	0.8248	0.6763	13.91
Moriches	54	0.7995	0.5847	16.73
Napeague	37	0.8013	0.5818	13.45
Shinnecock	66	0.7968	0.6214	17.27
LI	267	0.8174	0.6334	25.09

H_e: expected heterozygosity over all loci

H_o: observed heterozygosity over all loci

A: Allelic Richness (mean number of alleles per locus adjusted for sample size)

Table 3-4: Estimated number of effective breeders (*N_b*). *N_b* was estimated using the linkage disequilibrium method and 95% confidence interval estimated using jackknife method.

Location	<i>N_b</i>	95% CI
Cold Spring	141.4	111.6, 190.6
Hempstead	113.7	83.9, 173.3
Jamaica	65.3	25.3, 166.3
Moriches	262.5	189.0, 421.2
Napeague	88.0	71.6, 112.8
Shinnecock	289.7	219.9, 418.6
LI Bays	966.1	808.1, 1195.0

N_b: number of effective breeders
95% CI: 95% confidence interval.

Table 3-5: M-ratio calculated for all loci and sample location and overall sample locations.

	A441	J42	Pam21	Pam79	Pam27	WF12	WF6	WF33	WF32	WF16	WF27
Jamaica	0.47	0.89	0.33	0.67	0.41	0.5	0.58	0.46	0.2	0.39	0.23
Hempstead	0.71	0.74	0.33	0.29	0.41	0.83	0.66	0.43	0.29	0.47	0.37
Moriches	0.76	0.89	0.61	0.62	0.67	0.5	0.5	0.46	0.27	0.42	0.37
Shinnecock	0.65	0.84	0.61	0.57	0.52	0.83	0.67	0.46	0.34	0.36	0.5
Cold Spring	0.65	0.95	0.5	0.52	0.52	0.83	0.67	0.42	0.22	0.44	0.45
Napeague	0.76	0.68	0.44	0.45	0.52	0.83	0.5	0.34	0.2	0.36	0.33
overall	1	1	1	0.83	0.81	1	0.75	0.74	0.46	0.72	0.7

Table 3-6: Pairwise comparison of mean Internal Relatedness (IR) values per bay. Bolded values on the diagonal are the mean IR values of individuals sampled at the designated location, p-values of pairwise t-test are shown above the diagonal and significance level before Bonferroni correction is below the diagonal.

Average IR	Cold Spring	Hempstead	Jamaica	Morriches	Napeague	Shinnecock
Cold Spring	0.1532	0.5588	0.4461	0.0011	0.0015	0.0351
Hempstead	NS	0.1801	0.8488	0.0176	0.0164	0.2082
Jamaica	NS	NS	0.1900	0.0393	0.0340	0.3293
Morriches	**	*	*	0.2883	0.7956	0.1388
Napeague	**	*	*	NS	0.2995	0.1169
Shinnecock	*	NS	NS	NS	NS	0.2342

* $p < 0.05$

** $p < 0.01$

NS = not significant

Chapter 4

Genetic diversity and effective number of breeders for three Atlantic sturgeon (*Acipenser oxyrinchus*) populations

Introduction

Atlantic sturgeon (*Acipenser oxyrinchus*) is a large, long-lived anadromous fish which occurs from Labrador, Canada, to the St. John's River in Florida (Smith & Clingston 1997). Juveniles and spawning adults are found in approximately 20-35 rivers of the U.S.A. and Canada, while juveniles and non-breeding adults are found in estuaries and along the coast up to depths of 10-50 m (ASSRT 2007). Atlantic sturgeon have declined drastically since the late 1800s, mostly due to overfishing and habitat destruction (ASSRT 2007, Waldman & Wirgin 1998). They have a low intrinsic rebound potential due to their life history characteristics which include late maturity, low fecundity, infrequent spawning (females only spawn once every 1-5 years) and maximum egg production occurring at a late age (ASSRT 2007, Boreman 1997, Pikitch et al. 2005). Sturgeon exhibit strong philopatry to their natal rivers, making them vulnerable to localized stock depletion when anthropogenic pressures are spatially concentrated (Grunwald et al. 2008, King et al. 2001, Ong et al. 1996; Peterson et al. 2008; Waldman et al. 1996; Wirgin et al. 2000, 2002, 2012). Spawning population dynamics are largely independent between individual rivers and it is therefore critical to assess population parameters on a river-specific basis.

Our understating of the historic and contemporary spawning population sizes in different U.S. rivers is extremely limited for Atlantic sturgeon even though they have recently been listed under the U.S. Endangered Species Act (77 FR 5880, 77 FR5914), which mandates intensive management of this species. Historic spawning population sizes prior to 1890 for the Delaware and Hudson rivers were 180,000 and 6,000-6,800 mature females, respectively, while the Chesapeake Bay and its tributaries, including the James River supported a total of 20,000 adult females (ASSRT 2007, Secor 2002, Secor & Waldman1999). Over a century later, the Hudson River is currently estimated to contain 9,500 juvenile sturgeon in the estuary and the number of spawning adults using the river has been estimated at approximately 870 (600 males and 270

females, Kahnle 2007). Next to the Hudson the only other spawning population with a current size estimate is the Altamaha River ($N=343$ spawners). There is no current size estimate for the spawning population in the Delaware River, though it is known to be smaller than the population of the Altamaha River (i.e. <343 spawners). The James River is the only known spawning population contributing to the Chesapeake Bay and by the same logic is also thought to have less than 343 spawners. Both the Delaware River and James River have rebounded after coming very close to extirpation (Balazik et al 2010, Grogan & Boreman 1998, Secor & Waldman 1999). The total ocean population of Atlantic sturgeon (i.e., originating from all spawning rivers combined) was estimated to be 420,000 individuals between 2006-2011 (Kocik et al. 2013), with the Hudson and Delaware Rivers contributing approximately 50% and the James River contributing another 15%. This estimate is the census population size (N_c), which includes all breeding adults, subadults and juveniles that are not bound to rivers or estuaries during this period. The estimate was not broken down further by age group (Kocik et al. 2013).

Contemporary spawning population size estimates for the Hudson and Altamaha River were derived from sampling individuals in rivers and were based on tag-recapture techniques (Kahnle et al. 2007, Schueller & Peterson 2010). In fisheries management, genetic techniques are beginning to gain traction as a complementary tool for estimating various population parameters. The effective population size (N_e) is equivalent to the population size of an ideal population with no mutation, no selection, no migration and random mating, experiencing the same amount of genetic drift and inbreeding as the study population (Nunney 1993, Wright 1931). The number of breeders (N_b) is the number of breeding adults that produced individuals born during a specific period of time, for example, one spawning season. In a species with non-overlapping generations the generational N_e is equivalent to N_b of one spawning season. In species with overlapping generations N_e is frequently approximated by calculating the harmonic mean of N_b for multiple spawning seasons as N_b represents a subset of the number of potential spawners in that population (Nunney 1993, Palstra & Fraser 2012, Waples et al. 1990, Waples & Do 2009). Genetic estimation of N_e and N_b in wild populations is becoming increasingly common with the development of single-sample estimators that only need samples from one specific point in time as opposed to temporal methods that require samples taken several years apart (Hare et al. 2011, Leberg 2005, Luikart et al. 2010). The most commonly used single-sample estimator is based on the linkage disequilibrium (LD) method (Waples & Do 2008, 2009) because it requires only a

small sample size (30-50 individuals) and a moderate number of microsatellite loci (minimum 10). Here, the Key assumption is that the linkage disequilibrium signal resulting from recombination in small populations is driven solely by genetic drift. This method has been extensively reviewed and tested using simulations and widely applied to a variety of terrestrial, freshwater, anadromous and even a few marine species (Palstra & Fraser 2012, Waples & Do 2009). In addition to estimating these parameters, genetic techniques provide additional information on whether or not the spawning population is experiencing inbreeding, is losing genetic diversity to drift or has experienced a population bottleneck severe enough to influence its current genetic diversity.

Here, we use a multi-locus microsatellite analysis to estimate N_b of the Hudson, Delaware and James Rivers Atlantic sturgeon populations. We were able to accomplish this by sampling juvenile sturgeon concentrated at marine aggregation sites in the New York Bight and then assigning them back to their natal river of origin. We also assessed levels of inbreeding within these rivers and tested for evidence of population bottlenecks in their current genetic architecture. We used population genetic data and estimates of N_b to simulate genetic drift over time to assess the risk that these spawning populations will lose genetic diversity over the next century if their population size does not increase.

Methods

Sample acquisition & Laboratory procedures

Atlantic sturgeon are currently managed as five distinct population segments (DPS) (NMFS 2010). The Gulf of Maine (GOM) DPS is currently listed as threatened, the New York Bight (NYB), Chesapeake Bay (CB), Carolina and South Atlantic (SA) DPS as endangered under the Endangered Species Act (77 FR 5880, 77 FR5914). An alternative stock structure consisting of 6 DPS has been proposed based on larger sampling sizes and a combination of genetic data, life history characteristics, historical fisheries data and abundance trajectories (Grunwald et al. 2008). These DPS are based on natal rivers, though aggregations off the coast of New York and New Jersey (Dunton et al. 2010) have been shown to be comprised of a mixed stock along the entire coast.

Tissue samples were acquired during trawl surveys in the mid-Atlantic Bight as described in (Dunton et al. 2010, Dunton 2011). Individuals were captured at coastal aggregation sites for subadult Atlantic sturgeon occurring off the coast of Rockaway Peninsulæ, New York (Figure 4-

1) in May/June 2010, May 2011, October/November 2011 and May 2012. Trawls were performed in two general areas (approx. 3km long) approx 1.8km (40°33', 73°49') and 6.5km (40°31', 73°48') offshore. Sturgeon were measured, tagged and a fin clip was stored in 95% ethanol for genetic analysis. Genomic DNA was extracted from fin clips using the Qiagen Blood and Tissue extraction kit (Qiagen, Valencia, CA, USA). Twelve species-specific microsatellite loci previously successful for individual-based assignment of Atlantic sturgeon to their natal rivers and DPS of origin were amplified as described in (King 2001) and (Henderson-Arzapalo 2002). These microsatellites have from 3 to 21 alleles per locus and observed heterozygosities ranging from 0.3 to 1.

Data analysis

A mixed stock analysis was performed in ONCOR (Kalinowski et al. 2008) using previously established baseline data for ten river populations (Grunwald et al. 2008). We determined the genetic diversity of river populations for which we obtained an adequate number of individuals (> 30) by calculating the expected and observed heterozygosity, H_e and H_o (Nei 1978) and the allelic richness using FSTAT (Goudet 1995). We tested for linkage disequilibrium and conformity to Hardy-Weinberg Equilibrium (HWE) for each river using exact tests implemented in GENEPOP (Raymond & Rousset 1995, Raymond et al. 1997). We used two measures as indicators of inbreeding. At a sample level we calculated the inbreeding coefficient F_{IS} using FSTAT (Goudet 1995) and at an individual level we determined the internal relatedness (IR) as implemented in STORM (Frasier 2008). IR measures the level of relatedness of the parents of an individual. Outbred individuals will have a value equal to or below zero while positive values indicate the relatedness of the parents, with 1 being the maximum value.

We estimated the N_b for each river using the single-sample estimator LDNE. The linkage disequilibrium method is based on the small level of linkage of alleles that occurs due to sampling error during recombination (Waples 2006, Waples & Do 2008). The lowest included allele frequency was $p_{crit} = 0.02$. We used three tests to determine the presence of a genetic bottleneck, the M-ratio test (Garza & Williamson 2001) which is a strong indicator of recently occurring bottlenecks and the heterozygote excess test and mode shift test (Luikart et al. 1998) which can be calculated using Bottleneck (Piry et al. 1999). These test are all based on the assumption that rare alleles will be lost first during a population bottleneck.

To determine the likelihood of the Delaware, Hudson and James River populations losing genetic diversity over time, the loss of allelic richness was simulated across all loci using BOTTLESIM2.6 (Kuo & Janzen 2003). For each spawning population we simulated four scenarios using the determined allele frequencies as a starting point. For the first two simulations we assumed age at maturity = 20 years and longevity = 60 years and simulated the percent of alleles remaining assuming the population size to be equivalent to the upper or lower CI-boundary, respectively. For scenarios 3 and 4 we reduced the longevity to 10 years past age-at-maturity to simulate the effect of individuals not living out their natural lifespan due to anthropogenic factors. Assuming age at maturity = 20 years and longevity = 30 years, we simulated the loss of allelic richness for both the upper and lower CI boundary for each spawning population.

Results

A total of 460 individuals were genotyped. All individuals were immature and range in age from approximately 5-15 years old (personal communication K. Dunton). Mean total length of all recovered fish ranged from 75 – 207 cm, with a mean size of 120.5cm (std = 22.02cm). The frequency of size distribution for the three main contributing spawning populations is shown in Figure 4-2.

Individuals were assigned to their stocks of origin based on three proposed stock structures: ten natal river populations (Grunwald et al. 2008), the five DPS as listed (ASSRT 2007) and six DPS proposed by Grunwald et al. (2008). For the ten population mixed stock analysis approximately 95% of individuals were assigned to three populations: 83% were assigned to the Hudson River, 8% to the James River and 4% to the Delaware River. Individuals from the Ogeechee, Albemarle, Savannah and Kennebec River were sampled as well, though no individuals are assigned to the St. John, Edisto or Altamaha River (Figure 4-3A). Individuals from all 5 DPS were sampled (Figure 4-3B) with the majority of the individuals (87%) assigned to NYB, 8% to CB and 3% to the SE DPS with the remainder sampled from the Carolina and the GOM DPS. Similar distribution was found for the 6 DPS mixed stock analysis with 87% assigned to the NYB DPS, 8% to the CB DPS, 3% to the SE DPS and the remainder of the individuals assigned to the Carolina and Kennebec DPS. No individuals were assigned to the St. John DPS (Figure 4-3C).

Sufficient individuals were sampled originating from the Delaware (N=47), Hudson (N=322) and James River (N=36) populations to assess genetic diversity, test for inbreeding, estimate N_b

and determine the presence of genetic bottlenecks. Despite unequal sample sizes, similar levels of genetic diversity were estimated for all three populations (Table 4-1). Multi-locus heterozygosity H_o ranged from 0.66 – 0.69 and the allelic richness A ranged from 8.17 – 10.17 alleles per locus. Heterozygosity and number of alleles sampled at each locus is given in Table 4-1. No linkage disequilibrium was detected and all loci were in HWE with the exception of one locus (AO23) in one population (Hudson).

Multi-locus F_{IS} values were not significantly different from zero and with few exceptions single locus F_{IS} -values were also below or equal to zero (Table 4-1). Mean values of IR were low, 0.10 for both Delaware and Hudson River populations and 0.029 for the James River population with a standard deviation of 0.134-0.156 (Table 4-2). Maximum values of each population ranged from 0.316-0.596 equivalent to the parents being related at a level of half-siblings to siblings. In each population 10-12% of individuals had parents that were related at a level of half-siblings or higher. We determined N_b using the linkage disequilibrium method with $N_b = 62.1$ (95% CI = [44.3, 97.2]) for James River, $N_b = 108.7$ (95% CI = [74.7, 186.1]) for Delaware River and $N_b = 198.0$ (95% CI = [171.7, 230.7]) for the Hudson River population. The M -ratios calculated using global r are given in Table 3 for each locus, values ranged from 0.29 – 1.00 for Delaware River, 0.38-1.00 for Hudson River and 0.21 – 0.92 for the James River population. The mean multi-locus values were low ranging from 0.56 – 0.68, indicative of a recent or ongoing bottleneck.

The simulated loss of allelic richness over 100 years is shown in Figure 4-4. The percentage of alleles retained varied both by population and by the assumed population size (upper or lower range of estimated N_b). The percentage alleles remaining after a century in each simulated population ranged from 89.5-98.5% in the Delaware, 99 – 99.75% in the Hudson and 84.5-95% in the James River. For simulations run with a decreased longevity (30 years) the percentage of retained alleles was 0.6-4% lower, depending on the population, which differ in initial allele frequencies and assumed population size. In general the difference in loss of allelic richness between the two longevity scenarios was greater in the scenarios simulating the lower CI boundary for N_b .

Discussion

Mixed-stock analysis of the summer aggregations found in the NYB DPS assigned Atlantic sturgeon to all five listed DPS and further assigned individuals to seven of ten known

natal river populations implying the necessity of management of both natal estuaries and coastal aggregation sites for the recovery of Atlantic sturgeon. Even though these spawning populations are distributed along the entire US east coast, their migratory behavior leads them to be exposed to multiple threats, including habitat degradation and incidental by-catch in the small area in which subadults form aggregations.

Individual-based assignments using microsatellite data offer a higher resolution than using mitochondrial control region (mtCR) haplotypes to assign individuals to their spawning population of origin. Nevertheless, our data confirm a previous population level study of the same aggregations based on mtCR (Dunton et al. 2011) estimating the same proportional contributions of spawning populations from all listed DPS. Initial mixed-stock analysis of sturgeon aggregations in NYB in the 1990s using mitochondrial DNA assigned individuals almost exclusively to the Hudson River (Waldman et al. 1996). Subsequent analysis by Dunton et al. (2011) and results presented here indicate a much broader stock contribution. Similarly, assessments of sturgeon aggregations in Long Island Sound and the Connecticut River (Waldman 2012) have also demonstrated broad stock contributions. All of these studies suggest that southern DPS's of Atlantic sturgeon are recovering and contributing more individuals to marine aggregations along the coast than was evident two decades ago (Dunton et al. 2011; Waldman et al. 1996, 2012).

The Hudson River is the dominant contributor of sturgeon to marine aggregations in NYB, most likely due to its close proximity and because it is the largest remaining Atlantic sturgeon spawning population. Our results, however, suggest that the stock composition of marine aggregations is not always a function of size of the river's spawning stock and the proximity of the river to the aggregation site. The Delaware River has a larger spawning population and is closer to the coastal aggregations of the NYB, yet more NYB individuals were assigned to the James River than to the Delaware River. This result is unlikely to be the result of sampling or technical artifacts as the same pattern was identified by Dunton et al. (2010) in spring and fall. This unexpected result suggests that there are differences in marine migratory behavior between fish spawned in different rivers.

Understanding population size and trajectory is a critical part of endangered species management, yet we rarely have this information readily available for marine fish (Reynolds et al. 2005). Estimating the size of individual spawning populations of Atlantic sturgeon is

particularly difficult because subadults and adults migrate far from their spawning river and mix with sturgeon from other rivers in the coastal waters of the east coast of the United States. The long maturation time results in a delay of individuals returning to their natal river to spawn. This creates a lag that in turn makes it difficult to determine if a spawning population is responding to management. Using genetic methods I was able to use individuals sampled offshore in NYB assigned to spawning populations based on microsatellites to estimate N_b for two spawning populations of the NYB DPS (Hudson River, Delaware River) and one CB river (James River). At present, estimates of the spawning population size are only available for the Altamaha and Hudson Rivers, which were established using mark-recapture techniques. Despite only having collected sturgeon over a short sampling period (~ 3 years, 2010- 2012) I was able to determine N_b for a period of approximately 10 years because of the age range of sampled individuals (5 – 15 years). The number of effective breeders for the Hudson River was estimated to be 170-230 individuals. This number is smaller than the previous estimate of a total of 870 spawning adults, though remarkably similar to the estimate of 270 breeding females contributing to the Hudson River population (Kahnle et al. 2007, ASSRT 2007). This makes sense, because when there is an unequal sex ratio in the population any measure of N_b will skew towards the limiting sex, in this case females (Frankham 1996). The estimates of N_b presented here for the James and Delaware River represent the first estimates of spawning population size for these two rivers. Our estimates of 75-180 and 45-100 effective breeders for the Delaware and James River, respectively, confirm that these populations likely contain <300 spawners and will require intensive management if they are to continue to recover from near-extirpation (Balazik et al. 2010, Grogan & Boreman 1998, Secor & Waldman 1999).

The drastic decline of Atlantic sturgeon in the early 1900s resulted in a demographic bottleneck from which sturgeon have yet to fully rebound. The Hudson River spawning population was once the smallest of the three but is now the largest despite experiencing a decline of more than 95% (Kahnle et al. 2007, ASSRT 2007). The other two populations have experience declines on the order of 2-3 magnitudes (Kahnle et al. 2007, Secor 2002, Secor & Waldman 1999, ASSRT 2007). Despite these declines, only one of three tests employed detected a corresponding genetic bottleneck (the M -ratio test), though this is considered the most powerful of these tests to detect more recent or ongoing bottlenecks (Williamson-Natesan 2005). The M -ratio of all three spawning populations are below threshold values reported for other

species known to have suffered severe population declines and having very small extant populations (Garza & Williamson 2001). This indicates that Atlantic sturgeon have lost genetic diversity, in this case indicated by a loss of microsatellite alleles, but potentially also including adaptive genetic diversity as well (Franklin 1998; Lynch 1998). The Hudson is the least affected of the populations, while the James and Delaware exhibit similar low M -ratios. This is consistent with the known history of these three populations, with the James and Delaware declining to critically low levels and the Hudson faring slightly better (Franklin & Frankham 1998, Kahnle et al. 2007, Lynch & Lande 1998, Secor & Waldman 1999).

Inbreeding and genetic drift are the two main factors contributing to the loss of genetic diversity and the magnitude of their impact increases as N_e decreases. In each population inbred individuals were detected, although inbreeding was not high enough to cause deviations in Hardy Weinberg equilibrium at the population level. We detected 10-12% of individuals in each spawning population for which internal relatedness levels indicating that their parents were closely related, at a level of half-siblings or higher. Evidence for inbreeding in marine fishes has only been found in a handful of studies, in two flatfish species (Hoarau et al. 2005, O'Leary et al. 2013) and two shark species (Mourier & Planes 2013, O'Leary Chapter 5). Despite drastic differences in life history, these species and Atlantic sturgeon have in common that they display high levels of population structure and local fishing pressure can result in fragmented populations with increased non-random mating due to the limited availability of spawning partners as N_b decreases.

Genetic drift also poses a potential threat to the future genetic diversity of Atlantic sturgeon given that $N_e > 500$ and $N_e > 50$ have been suggested as thresholds above which populations are able to maintain adaptive potential and have a low risk of inbreeding, respectively (Franklin & Frankham 1998, Lynch & Lande 1998). Estimates of N_b for all three spawning populations assessed are below the range of these critical population sizes, suggesting a need to assess the risk that genetic drift now poses to their genetic diversity. Our simulations show that these populations are robust to genetic diversity loss, assuming that N_b stays the same or grows over that period. The Hudson River spawning population is projected to retain >98 % of its alleles, whereas the Delaware and James River spawning populations are both likely to retain >80-90% of their alleles. We found that genetic diversity loss was most severe as longevity decreased in these populations, suggesting a need to minimize mortality of mature

Atlantic sturgeon. A long generation time can result in a persistent bottleneck having a shortened effective time and can act as an intrinsic buffer against the loss of genetic diversity resulting in a 'genetic storage' effect (DeHaan et al. 2006, Lippe et al. 2006). Chapman et al. (2011) found a similar effect in smalltooth sawfish (*Pristis pectinata*) that have unexpectedly high levels of genetic diversity despite having experienced a drastic decline in population size. Similar to our simulations projecting the loss of genetic diversity in sturgeon over the next century, Chapman et al. (2011) found that the long-lived small-toothed sawfish will most likely retain 90% of their current genetic diversity despite a relative small N_e . Other long-lived animals have also been shown to be similarly robust to genetic diversity loss. Examples include fish (e.g. the copper redhorse, Lippe et al. 2006), mammals (e.g. kangaroos, Busch et al. 2007, rhinoceros, Dinerstein et al. 1990), reptiles (e.g. box-turtles, Kuo et al. 2004), and birds (e.g. eagles (Hailer et al. 2006; Lerner et al. 2009).

Genetic approaches can contribute valuable information regarding N_e and recent trajectory in endangered species, yet are not as commonly used in assessing marine and anadromous species as they are in assessing terrestrial species (Hare et al. 2011, Waples & Do 2009). Our study confirms a few previous studies indicating that marine aggregations of immature Atlantic sturgeon contain a mix of individuals from different spawning rivers. Although size of the spawning population and proximity to the aggregation site are important determinants of each river's contribution to the marine aggregation, it is likely that other factors are involved as well. Nevertheless, the reappearance of southern sturgeon in NYB aggregations suggests some recovery of these populations. We also find that N_b for ~10 years in the early 2000s for each river is measured in the hundreds, with the Hudson being the largest. All three populations we genetically assessed exhibit evidence of a recent bottleneck, with the Hudson being the least affected of the three, and about 10% of individuals are inbred. Our simulations suggest that while these populations are small, the longevity of sturgeon will enable them to retain most of their genetic diversity for the next century, especially if the N_e grows. Our analyses indicate that stringent management efforts for Atlantic sturgeon focused on threats in both rivers and the ocean remain important for the recovery of this iconic species.

Figure 4-1: Map of Atlantic sturgeon sampling locations and natal rivers. Arrow indicates sample area off Rockaway, NY. Trawls were performed in two general areas (approx. 3km long) approx. 1.8km ($40^{\circ}33'$, $73^{\circ}49'$) and 6.5km ($40^{\circ}31'$, $73^{\circ}48'$) offshore. The three main natal rivers (Delaware, Hudson, James) contributing to aggregations of subadults in New York Bight are depicted.

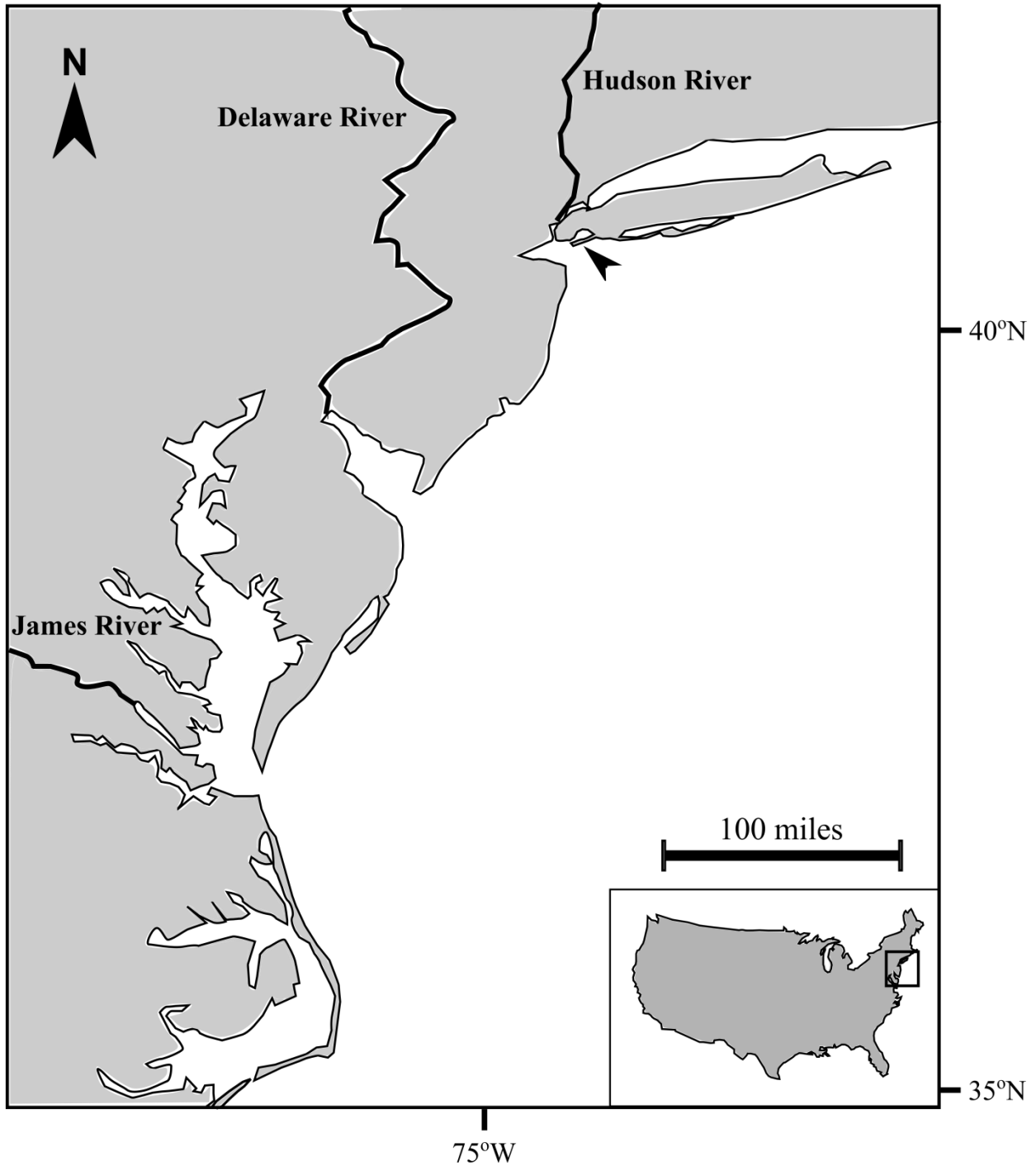


Figure 4-2: Frequency distribution of total length [cm] of recovered individuals. Proportion of individuals assigned to Delaware (black), Hudson (grey) and James (white) river for each size category shown.

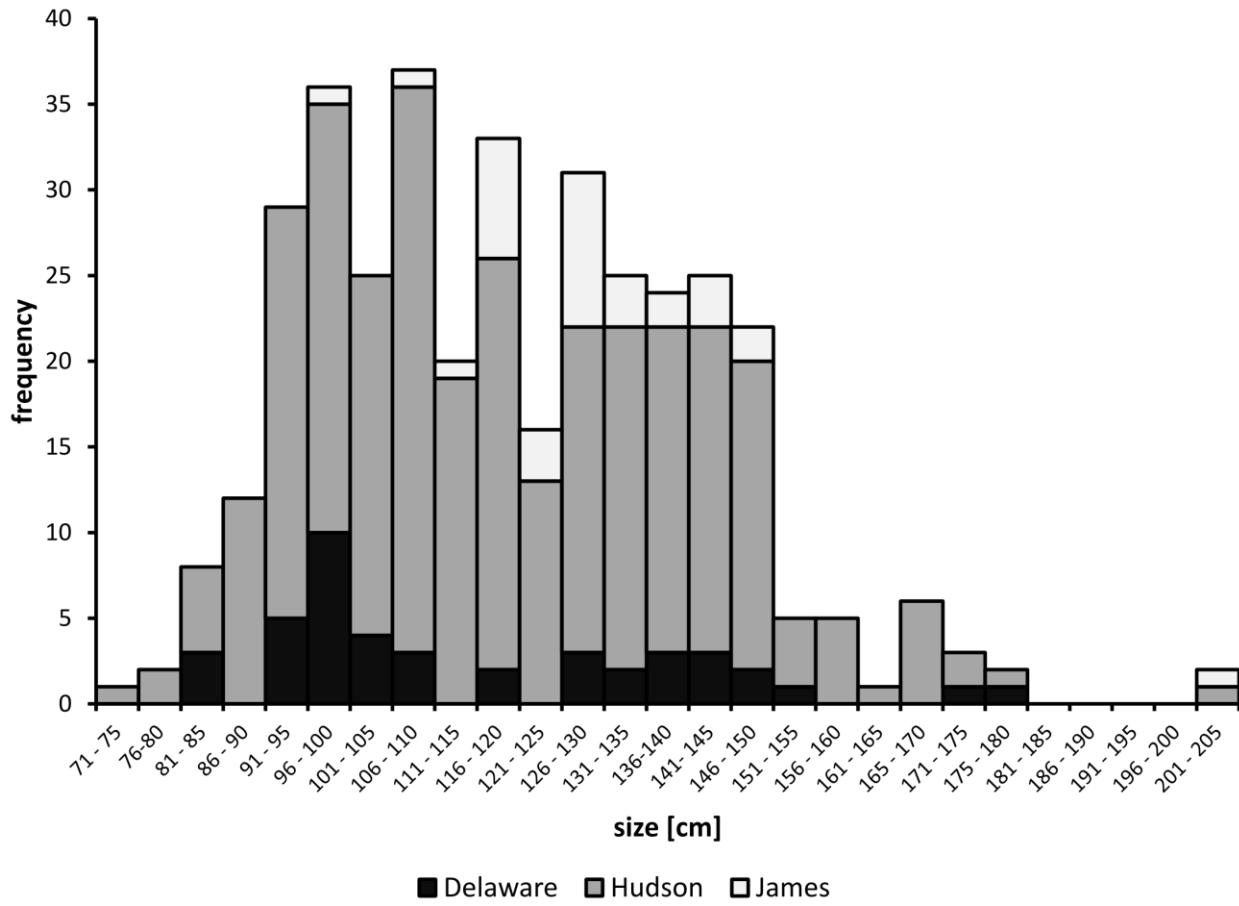


Figure 4-3: Proportion of individuals assigned to stocks. Individuals are assigned to populations based on microsatellite genotype. (A) Mixed stock analysis (MSA) based on 10 river spawning populations. (B) MSA based on 5 distinct population segments (DPS) listed by the National Marine Fisheries Service (NMFS). (C) MSA based on 6 DPS suggested by Grunwald et al. 2008. No individuals were assigned to St. John DPS.

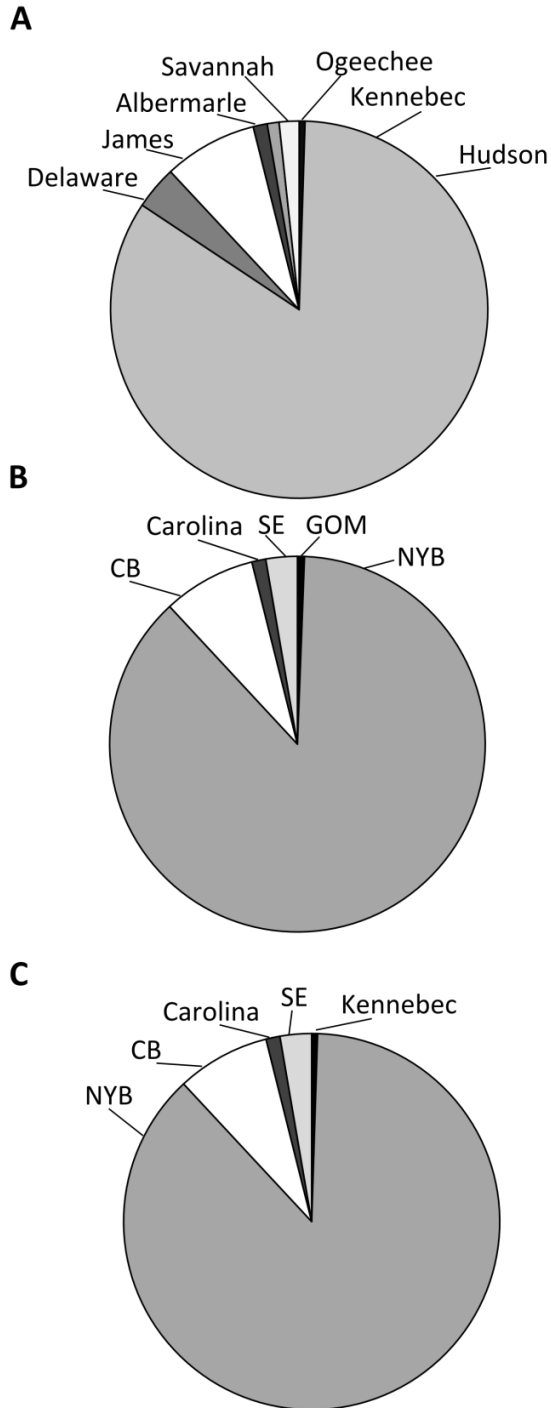


Figure 4-4: Simulation of loss of allelic richness over 100 years. Results show percentage of remaining alleles each year over 100 years for upper and lower CI boundaries of estimated population size for Delaware (Del180, Del75), Hudson (Hud230, Hud170) and James (Jam100, Jam40) Rivers should N_e remain constant. (A) Age at maturity = 20years, longevity = 60years. (B) Age at maturity = 20 years, longevity = 30 years.

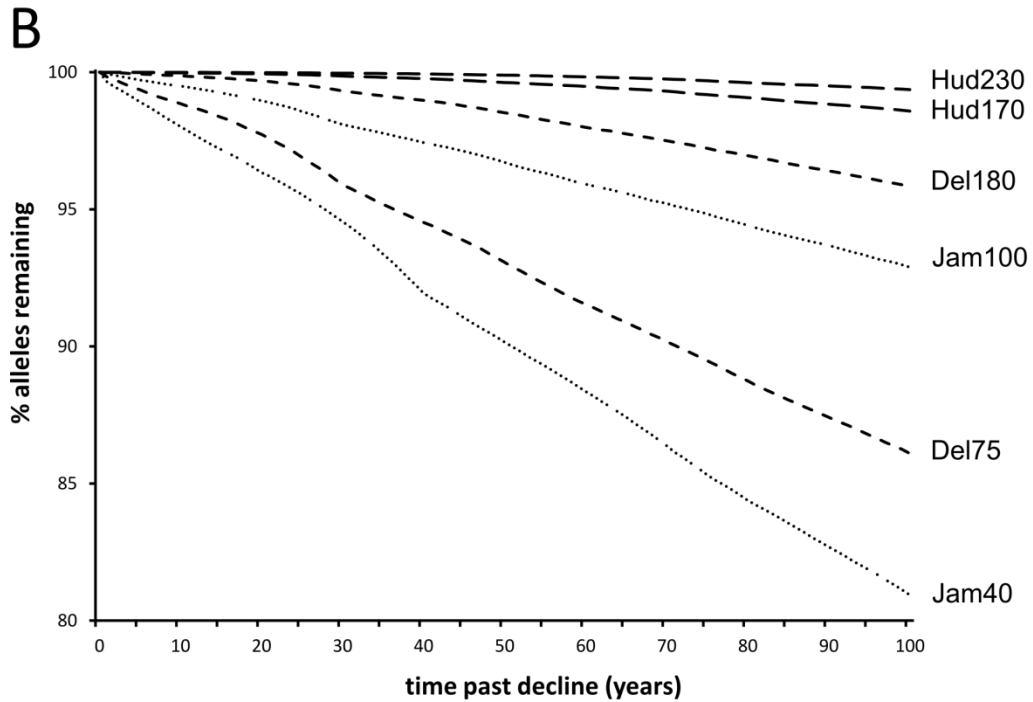
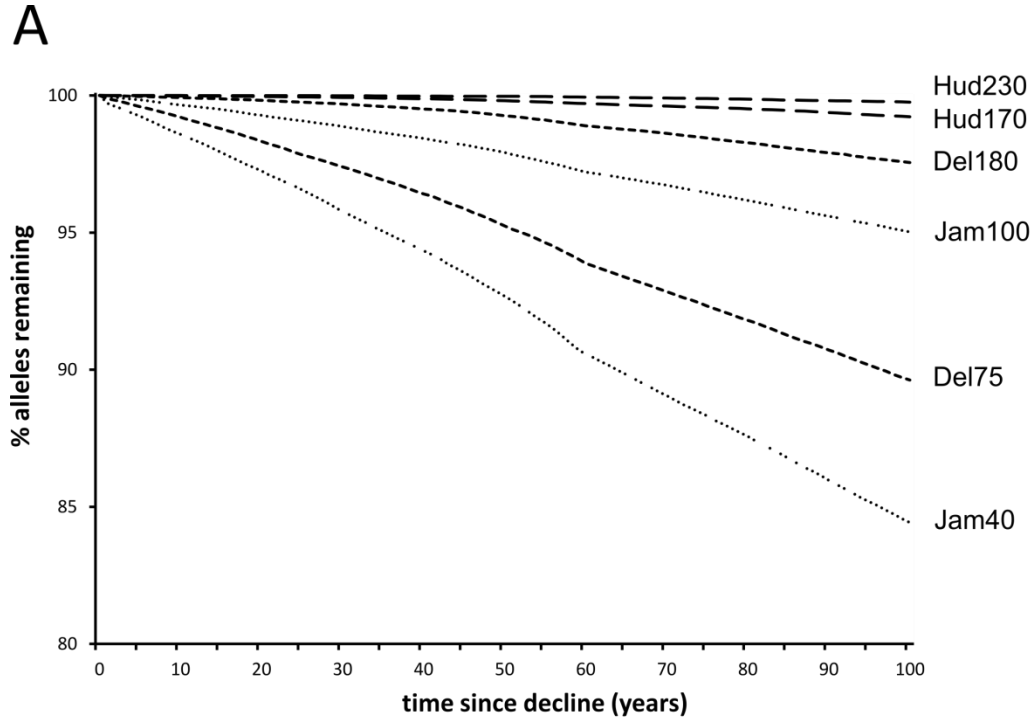


Table 4-2: Genetic diversity of Atlantic sturgeon. Multi-locus and single-locus comparisons of genetic diversity between Delaware, Hudson and James River samples.

	Delaware	Hudson	James	Total no. alleles
N	47	322	36	
H _e	0.666	0.677	0.694	
H _o	0.675	0.677	0.712	
A	8.33	10.17	8.17	
F _{IS}	-0.015	0.000	-0.027	
LS68				
H _e	0.695	0.720	0.826	
H _o	0.681	0.699	0.886	
A	5	6	9	9
F _{IS}	0.02	0.029	-0.073	
LS19				
H _e	0.559	0.610	0.417	
H _o	0.447	0.581	0.417	
A	5	6	3	6
F _{IS}	0.203	0.047	0.002	
LS39				
H _e	0.042	0.034	0.236	
H _o	0.043	0.034	0.229	
A	2	4	3	5
F _{IS}	-0.011	-0.011	0.03	
LS54				
H _e	0.386	0.335	0.229	
H _o	0.404	0.345	0.250	
A	3	3	3	3
F _{IS}	-0.047	-0.028	-0.094	
AO23				
H _e	0.689	0.691	0.779	
H _o	0.745	0.649	0.861	
A	9	9	6	10
F _{IS}	-0.081	0.061	-0.107	
AO45				
H _e	0.809	0.870	0.869	
H _o	0.872	0.894	0.944	
A	9	11	11	12
F _{IS}	-0.079	-0.028	-0.088	
AO12				
H _e	0.834	0.824	0.854	
H _o	0.830	0.839	0.861	
A	12	16	11	17
F _{IS}	0.005	-0.018	-0.008	
D170				
H _e	0.850	0.815	0.803	
H _o	0.872	0.836	0.771	
A	9	9	7	9
F _{IS}	-0.026	-0.027	0.04	
D188				
H _e	0.685	0.717	0.867	

H_o	0.681	0.702	0.857	
A	11	15	12	17
F_{IS}	0.006	0.021	0.012	
D165				
H_e	0.720	0.783	0.714	
H_o	0.723	0.795	0.722	
A	8	9	8	9
F_{IS}	-0.005	-0.016	-0.012	
D44				
H_e	0.855	0.830	0.883	
H_o	0.915	0.847	0.889	
A	11	13	12	14
F_{IS}	-0.071	-0.021	-0.007	
D241				
H_e	0.861	0.902	0.853	
H_o	0.894	0.904	0.861	
A	16	21	13	22
F_{IS}	-0.038	-0.002	-0.01	

N: Sample size

H_e : expected heterozygosity

H_o : observed heterozygosity

A: allelic richness (number of alleles per locus)

F_{IS} : inbreeding coefficient

* deviation from HWE ($p < 0.05$)

Table 4-3: Internal relatedness (IR) of Atlantic sturgeon natal river populations. Mean values of IR for Delaware, Hudson and James River samples.

	Delaware	Hudson	James
IR	0.010	0.010	0.029
SD	0.144	0.156	0.134
minimum	-0.306	-0.319	-0.224
maximum	0.353	0.596	0.316
% over 0.2	10	12	11

IR: Internal Relatedness

SD: Standard deviation

Minimum: minimum value

Maximum: maximum value

% over 0.2: % of individuals within samples with IR over 0.2

Table 4-3: Results of M-ratio test for bottlenecks. M-ratios calculated using global r for Delaware, Hudson and James River samples for each locus and mean multi-locus values.

	Delaware	Hudson	James
LS68	0.31	0.38	0.56
LS19	0.36	0.43	0.21
LS39	0.29	0.58	0.43
LS54	0.43	0.43	0.43
AO23	0.69	0.69	0.46
AO45	0.75	0.92	0.92
AO12	0.42	0.55	0.39
D170	1.00	1.00	0.78
D188	0.46	0.63	0.50
D165	0.89	1.00	0.89
D44	0.55	0.65	0.60
D241	0.70	0.91	0.57
mean	0.57	0.68	0.56

Chapter 5

DNA profiling reveals heightened vulnerability of white sharks, *Carcharodon carcharias*

Introduction

Understanding the contemporary size and recent trajectory of marine fish populations remains a difficult problem and has major economic and environmental implications (Hare et al. 2011, Reynolds et al. 2005). Genetic analysis can provide pivotal information about the size and recent history of severely depleted populations and is frequently used in conservation planning for terrestrial endangered species (Frankham 1996). Recent analytical advances have also enabled estimation of the effective number of breeders (N_b) in small populations, a management-relevant parameter approximating the number of reproducing adults (Hoehn et al. 2012, Palstra & Fraser 2012, Waples & Do 2009). However, a variety of obstacles have slowed the integration of these types of analyses into the conservation management of wild marine fish populations (Hare et al 2011, Waples & Do 2009). Marine fish have relatively large populations compared to most terrestrial animals, which both hinders estimation of N_b and makes them more resilient to processes influencing the genetic architecture of very small populations (e.g., genetic drift, inbreeding) (Ward et al. 1994, DeWoody & Avise 2000).

White sharks (*Carcharodon carcharias*) are iconic apex predators and one of the world's most legislatively protected marine fish species (Compagno et al. 1997). They primarily occur in seven regions: the Northwest Atlantic, Northwest Pacific, Northeast Pacific, Australasia, southern Africa, South America and Mediterranean (Compagno et al. 1997) and several of these populations have likely declined due to overfishing (Dulvy et al. 2008). There have been 3 attempts to estimate regional white shark population sizes, all involving mark-recapture methods (Chapple et al. 2011, Cliff et al. 1996). Chapple et al. (2011) reported that the number of subadult and adult white sharks off Central California was 130-275 individuals (2006-2008), although Sosa-Nishizaki et al. (2012) detailed how several assumptions of the mark-recapture model were violated and suggested this was an underestimate. In South Africa, Cliff et al. (1996) estimated that the population consisted of 1,279 individuals (C.V. 24%) between the Western Cape and Richards Bay, the geographic extent of the shark beach meshing program where tagged

sharks were potentially recaptured. Another tag-recapture study using photographic identification methods conducted in Gansbaai between 2007-2011 estimated that the population size for the same region as Cliff et al. (1996) eleven years later was 808–1008 individuals (Towner et al. 2013). Although white shark population estimates are few, difficult to obtain and likely to generate controversy given uncertainties about how they are calculated, it does appear that regional populations are relatively small.

Genetic data has been collected for white sharks in many regions and primarily used to examine regional population structure (Blower et al. 2012, Gubili et al. 2010, Jorgensen et al. 2010, Pardini et al. 2001, Tanaka et al. 2011). Little attention has been paid to levels of intrapopulation genetic diversity of this species and what that information suggests about regional population sizes and trajectories. Genetic approaches may provide complementary information useful for understanding the status of white shark populations. Here, the patterns of genetic diversity among white sharks sampled in the Northwest Atlantic and southern Africa is reported. The isolation, inbreeding levels and assessed potential genetic bottlenecks in these regions are examined using mitochondrial control region (mtCR) sequences and 14 microsatellite loci. Further, N_b is estimated for each region during the sampling period.

Materials and Methods

Individual white sharks were sampled from 2001 - 2008 in the Northwest Atlantic (N=31) and 2003 - 2010 in southern Africa (N=27). All of the individuals were immature (<3.0m total length, Compagno et al. 1997). Genomic DNA (gDNA) was extracted from 0.015 – 0.035g of tissue using the Qiagen Blood and Tissue extraction kit (Qiagen, Valencia, California, USA). The mtCR was amplified using species-specific primers and modified PCR protocol as described in Blower et al. (2012) and sequenced on a 3730 DNA Analyzer (Life Technologies). Forward and reverse mtCR sequences were individually checked for quality and aligned using ClustalX (Thompson et al. 2002) along with haplotypes from previous studies: HQ414073-HQ414086 (Blower et al. 2012), AY026196-AY026224 (Pardini et al. 2001), GU002302-GU00232 (Jorgensen et al. 2010), HQ540294-HQ540298 (Gubili et al. 2010). The alignment was then trimmed to 826 base pairs and exported into ARLEQUIN v3.5 (Excoffier & Lischer 2010) to characterize haplotypes using the Tamura & Nei model and calculate pairwise Φ_{ST} and a minimum spanning tree, which was visualized using HAPSTAR (Teacher & Griffiths 2011).

Individuals were genotyped at 14 polymorphic microsatellite loci (Gubili et al. 2010, Shrey & Heist 2002, O'Leary et al. 2013) (Table 5-1). Amplicons were sized on a 3730 DNA Analyzer (Life Technologies) using an internal size standard (500 LIZ™, Life Technologies) and scored using Peakscanv1.0 (Applied Biosystems). Eight homozygotes per locus were re-amplified at ($T_a - 3^\circ\text{C}$) to expose possible null alleles. Approximately 40% of all genotypes were re-scored by a second analyst to avoid genotyping error. Quality control was performed using MICROSATELLITE TOOLKIT FOR EXCEL (Park 2001) to detect scoring errors, duplicate genotypes, and MICROCHECKER (van Oosterhout et al. 2004) to determine large allelic dropouts, null alleles and large allelic gaps. All statistics were calculated including all loci and after having removed loci that were out of Hardy-Weinberg Equilibrium (HWE), though differences were negligible. Population differentiation was determined using pairwise F_{ST} and Bayesian clustering. STRUCTURE (Pritchard et al. 2000) was run for a burn-in period of 15,000 MCMC steps followed by 350,000 MCMC steps using the admixture model (with/without a priori location) for $K = 1-5$ for 10 independent runs each to determine convergence.

The genetic diversity in each region was assessed by calculating the haplotype and nucleotide diversities of the mitochondrial haplotypes using ARLEQUIN v3.5 and the expected and observed heterozygosity and allelic richness of the microsatellite loci using FSTAT (Goudet 1995). We used exact tests implemented in GENEPOP (Raymond & Rousset 1995, Raymond et al. 1997) to determine deviations from HWE and linkage disequilibrium. Levels of inbreeding were determined using two measures: the inbreeding coefficient F_{IS} , calculated using FSTAT, which measures intrapopulation heterozygote deficiency and the internal relatedness (IR) using STORM (Frasier 2008), which measures the relatedness of an individuals' parents (Amos et al. 2001). For outbred individuals IR-values should be below or close to zero. For parents that share many alleles (i.e. are closely related) IR should be positive, with 1 being the maximum value.

The number of effective breeders was determined using the linkage disequilibrium method as implemented in LDNE (Waples & Do 2008). This number is closest to N_b producing the specific cohorts sampled individuals belong to, as the sample period is shorter than the generation time of this species. We tested for bottlenecks using the mode-shift test, heterozygosity excess test (Piry et al. 1999) and M-ratios (Garza & Williamson 2001). The M-ratio calculates the ratio of extant alleles in a population (k) to the total number of alleles expected given the range of allele sizes (r). The loss of rare alleles during population bottlenecks

tends to affect k more than R , thus reducing the ratio. We only used loci with 5 or more alleles for this calculation ($N=11$ loci).

Results and Discussion

White shark populations in the Northwest Atlantic and southern Africa are genetically isolated. Maternally inherited mitochondrial DNA sequences ($\Phi_{ST} = 0.10$, $p < 0.00001$) and biparentally inherited microsatellite loci ($F_{ST} = 0.064$, $p < 0.00001$) were both strongly partitioned between these regions. Bayesian clustering of the microsatellite data also revealed two distinct clusters corresponding to individuals from the Northwest Atlantic and southern Africa, respectively (Figure 5-1A). Both study populations were also differentiated from Mediterranean, Australasian and North Pacific populations based on previously published mitochondrial DNA sequences (Blower et al. 2012, Gubili et al. 2010, Jorgensen et al. 2010, Pardini et al. 2001) with no shared haplotypes between them, with the exception of one shared haplotype between the Northwest Atlantic and southern Africa (Figure 5-1B). White sharks in the Northwest Atlantic and southern Africa were therefore treated as separate, isolated populations for all subsequent analyses.

Small, isolated populations are vulnerable to inbreeding as reproducing adults have an elevated probability of mating with relatives (Wright 1922, Wright 1984). Non-random mating of this nature generates deviations in allele frequencies from theoretical expectations in the form of deficiencies in heterozygotes. We observed chronic heterozygote deficiencies in both populations that are unlikely to result entirely from technical or sampling artifacts. Null alleles occur when there are point mutations in the flanking regions of microsatellite loci where the primers bind, resulting in non-amplification of an allele and false positive homozygotes. Lowering the annealing temperature reduces the binding stringency; re-amplifying homozygous individuals at lower annealing temperatures did not result in additional alleles ruling out technical artifacts. Wahlund effect, the inadvertent sampling of more than one population as one was ruled out by testing for temporal and spatial population structure. Further, re-calculating various statistics excluding markers out of HWE did not result in significantly different values (Table 5-1). Multi-locus inbreeding coefficients (F_{IS}) were 0.18 and 0.19 for southern Africa and the Northwest Atlantic, respectively (Table 5-1). The mean internal relatedness (IR) for the Northwest Atlantic and southern Africa populations was 0.22 (std = 0.26, range = -0.22 – 0.56)

and 0.21 (std = 0.16, range = -0.17 – 0.45), respectively. This indicates that mating pairs frequently exhibit co-ancestry and are related at a level approaching that of half-siblings.

The estimate for N_b in southern Africa had an unbound upper confidence interval ($N_b = 92.5$; 95% C.I. 28.8- ∞), indicating that N_b during the study period was too high to estimate using this method with the available sample size. In contrast, N_b had narrow confidence intervals and was remarkably small for the Northwest Atlantic: 29 individuals (95% C.I. 21-49). Two thirds of the Northwest Atlantic individuals analyzed were captured in 2001 - 2003 and were <2.5 m in length at the time, meaning the parameter we estimated was likely closest to N_b producing these particular cohorts. In the absence of information on finer scale population structure and the breeding frequency of adult white sharks it is difficult to reconcile N_b estimated for this defined period with the actual numbers of adults alive at the time. Nevertheless, the N_b range estimated for Northwest Atlantic white sharks is remarkably small, approaching those of mammalian and avian apex predators known to exist in very small, isolated populations, e.g. polar bears, *Ursus maritimus*, (Cronin et al. 2009), Madagascar fish eagle, *Haliaeetus vociferoides* (Johnson et al. 2009), puma, *Puma concolor* (Miotto et al. 2011).

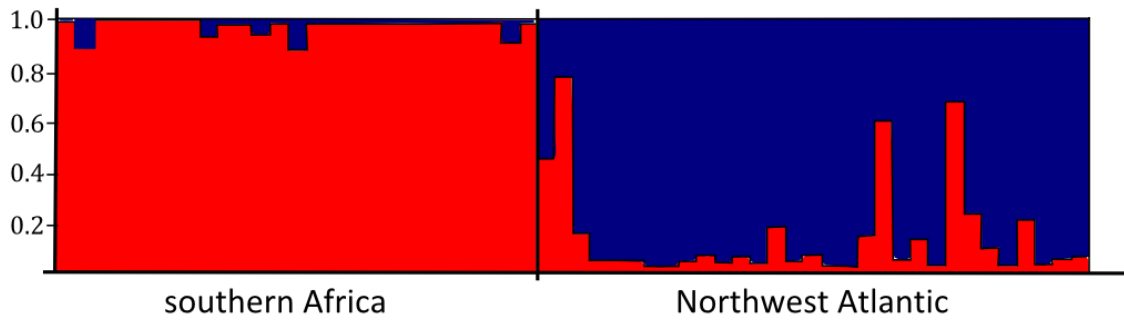
Mitochondrial haplotype diversity (0.7237 and 0.7495, for SA and US respectively) and nucleotide diversity (0.0059 and 0.0031, for SA and US respectively) were not particularly low in these regions when compared to other shark populations (Hoelzel et al. 2006 and species cited therein), although the Northwest Atlantic population exhibits the “starburst” pattern of haplotype relationships that is usually associated with historic bottlenecks or founder effects (Avice 2000). Two of the tests failed to detect evidence of a bottleneck. The mode shift test showed a normal L-shape distribution and excess homozygosity was not significant in either population ($p_{SA}=0.46$, $p_{US}=0.38$). The M-ratio test, however, indicated there were “lost alleles” in each population, which is consistent with a recent genetic bottleneck. One interesting observation was that M-ratios were sensitive to the choice of allelic size range (r) used. When we use the range derived from the alleles observed in both regions combined the mean M-ratios for southern Africa and the Northwest Atlantic were 0.59 and 0.58, respectively, which is well below what is typical for bottlenecked populations (Garza & Williamson 2001). When using the size range of alleles observed within each region, the mean M-ratios were higher (0.73 and 0.70, respectively) but still relatively low compared to most non-bottlenecked populations (Garza & Williamson 2001). Garza & Williamson’s original description of the M-ratio test does not specify which allelic

range to use (species or population), but we would argue that the population-specific r is likely to be the most conservative.

These findings indicate that some white shark populations exhibit characteristics more typically seen in small, fragmented populations of terrestrial or freshwater species, such as modest N_b , high levels of inbreeding or recent bottlenecks. In fact, the white shark population in the Northwest Atlantic is small enough that it is possible to estimate N_b for a small number of cohorts using genetic methods. These genetic characteristics have previously been described in a handful of marine fish typically characterized by a broadcast spawning reproductive mode (Type III survivorship) and large variance in reproductive success (Hauser et al. 2002, Hoarau et al. 2005, O'Leary et al. 2013b). This study indicates that some highly mobile sharks, with the contrasting life-history pattern to these fish, exhibit similar characteristics. Emerging studies of other sharks and rays are also finding evidence of inbreeding, recent bottlenecks or modest N_b (Chapman et al. 2011, Hoelzel et al. 2006, Mourier & Planes 2013), although none have detected evidence of all three, as found here in white sharks. Routine analysis of intrapopulation genetic diversity, which is a staple of conservation planning for terrestrial and freshwater species, can therefore also yield important information for the conservation of imperiled shark populations.

Figure 5-1: Population structure of white sharks using nuclear and mitochondrial markers. (A) Bayesian inference of genetic structure. Sample locations are indicated on the x-axis. Each individual is represented by a single vertical column with the y-axis indicating the membership coefficient for one of two clusters. (B) Haplotype network of the mitochondrial control region. Haplotypes from South Africa and the Northwest Atlantic are scaled based on frequency of occurrence in the sample set.

A



B

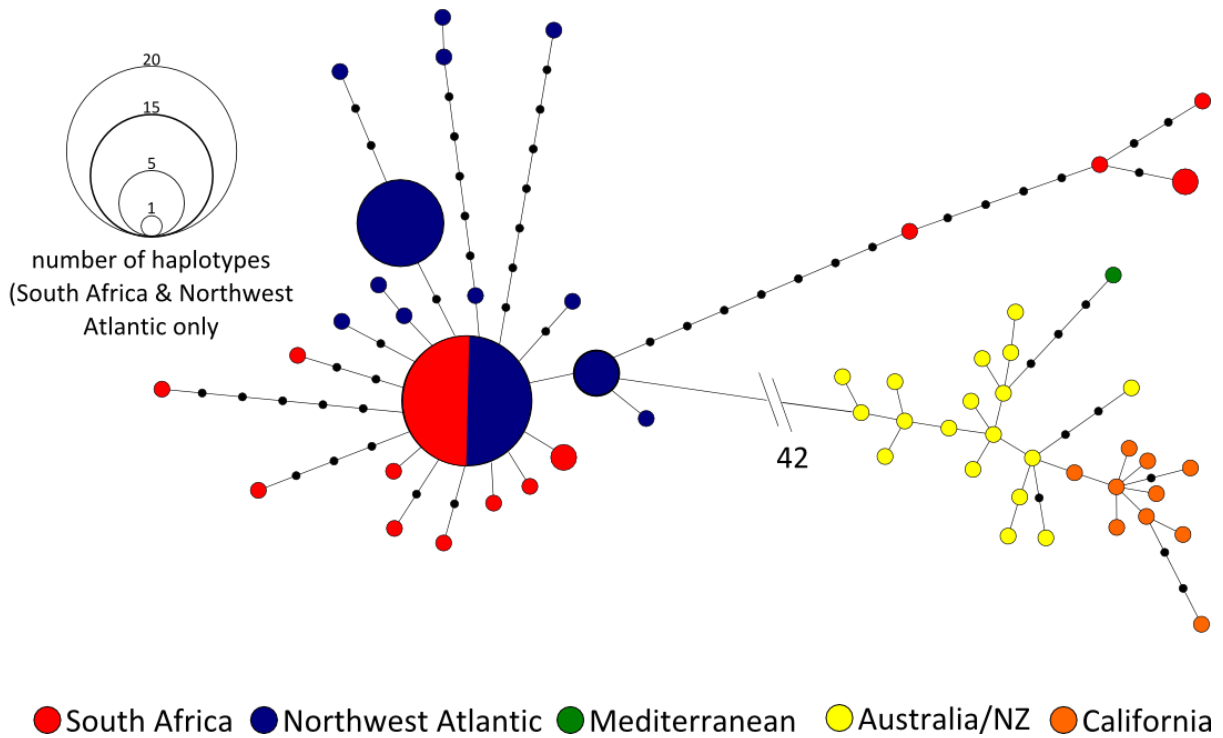


Table 5-1: Genetic diversity of South African and Northwest Atlantic populations.

	South Africa	USA		South Africa	USA
CCa9			CCa1466		
<i>He</i>	0.824	0.725	<i>He</i>	0.498	0.553
<i>Ho</i>	0.826	0.607**	<i>Ho</i>	0.346	0.516
<i>A</i>	11	10	<i>A</i>	3	4
<i>F_{IS}</i>	-0.002	0.165	<i>F_{IS}</i>	0.31	0.067
<i>M-ratio</i>	0.61 (0.85)	0.56 (0.77)	<i>M-ratio</i>	0.67 (1.00)	0.67 (0.67)
CCa1077			CCa711		
<i>He</i>	0.797	0.802	<i>He</i>	0.722	0.764
<i>Ho</i>	0.407***	0.733	<i>Ho</i>	0.5**	0.69
<i>A</i>	8	10	<i>A</i>	6	9
<i>F_{IS}</i>	0.493	0.087	<i>F_{IS}</i>	0.313	0.099
<i>M-ratio</i>	0.57 (0.57)	0.71 (0.71)	<i>M-ratio</i>	0.26 (0.60)	0.39 (0.53)
Cca 6.27			Cca1273		
<i>He</i>	0.73	0.865	<i>He</i>	0.444	0.557
<i>Ho</i>	0.5*	0.833	<i>Ho</i>	0.4	0.308***
<i>A</i>	6	10	<i>A</i>	3	5
<i>F_{IS}</i>	0.319	0.037	<i>F_{IS}</i>	0.101	0.453
<i>M-ratio</i>	0.60 (0.67)	1.00 (1.00)	<i>M-ratio</i>	0.43 (0.60)	0.71 (0.71)
CCa1226			CCa1419		
<i>He</i>	0.693	0.766	<i>He</i>	0.819	0.631
<i>Ho</i>	0.4*	0.367***	<i>Ho</i>	0.84**	0.655
<i>A</i>	6	6	<i>A</i>	8	5
<i>F_{IS}</i>	0.428	0.525	<i>F_{IS}</i>	-0.026	-0.039
<i>M-ratio</i>	0.67 (1.00)	0.67 (0.86)	<i>M-ratio</i>	0.89 (0.89)	0.56 (0.63)
CCa1536			CCa418		
<i>He</i>	0.854	0.76	<i>He</i>	0.445	0.15
<i>Ho</i>	0.652***	0.613**	<i>Ho</i>	0.35	0***
<i>A</i>	11	10	<i>A</i>	3	2
<i>F_{IS}</i>	0.241	0.196	<i>F_{IS}</i>	0.218	1
<i>M-ratio</i>	0.52 (0.55)	0.48 (0.53)	<i>M-ratio</i>	0.50 (0.50)	0.33 (1.00)
CCa85			m59^		
<i>He</i>	0.856	0.771	<i>He</i>	0.725	0.655
<i>Ho</i>	0.722	0.593	<i>Ho</i>	0.667	0.5
<i>A</i>	8	6	<i>A</i>	8	6
<i>F_{IS}</i>	0.44 (0.44)	0.33 (0.38)	<i>F_{IS}</i>	0.081	0.24
<i>M-ratio</i>			<i>M-ratio</i>	0.73 (0.89)	0.55 (0.67)
CCa1276			IoX10		
<i>He</i>	0.899	0.773	<i>He</i>	0.669	0.603
<i>Ho</i>	0.783*	0.667*	<i>Ho</i>	0.813	0.5
<i>A</i>	13	10	<i>A</i>	5	4
<i>F_{IS}</i>	0.132	0.14	<i>F_{IS}</i>	-0.223	0.174
<i>M-ratio</i>	0.68 (0.81)	0.53 (0.53)	<i>M-ratio</i>	0.71 (0.83)	0.57 (0.80)

significant deviations from HWE: * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$)

He/Ho: expected/observed heterozygosity

A: number of alleles per locus in the region sampled.

F_{IS}: inbreeding coefficient

M-ratio per locus and population calculated using global *r* and (population *r*).

^unpublished locus

Chapter 6

Genetic estimation of effective population size and detection of recent bottlenecks and inbreeding in marine fish populations: a review.

Introduction

Genetic variation is the fundamental building block of biodiversity and harbors the adaptive evolutionary potential that enables species to persist in an ever changing environment (May 1994, McNeely et al. 1990, Willi et al. 2006). As such, the loss of genetic diversity within populations and species has been recognized as a major issue in the conservation and management of terrestrial and freshwater organisms, though it has by and far been neglected as a management concern for marine species (Frankham 1996, Kenchington & Heino 2002). Genetic diversity loss occurs as a result of several processes, including the stochastic loss of variation occurring as it is transmitted between generations (“genetic drift”), population bottlenecks and non-random mating (Allendorf 1986, Wright 1922, 1931, 1984). The effective population size (N_e) is the number of individuals in an idealized population that is experiencing the same amount of genetic drift as the study population, i.e., it is the number of reproductively mature adults from one generation effectively contributing to the next generation (Wright 1922).

Demographic bottlenecks occur when a population experiences and then recovers from a sudden decline in abundance. Bottlenecks cause a temporary reduction in N_e and can therefore reduce genetic diversity of the population (England et al. 2003, Frankham et al. 1999, Nei 1975).

Inbreeding is a form of non-random mating within a population in which mating partners are more closely related to one another than expected by chance (Wright 1922, 1931). Inbreeding is

most common in severely reduced populations and when dispersal from the natal site is low (Keller & Waller 2002).

Large populations that have corresponding large N_e are less vulnerable to genetic drift, bottlenecks and inbreeding than small populations. Marine fish have been long thought to exist in large, widely distributed populations that are connected over wide geographic scales by movement and reproductive mixing (Hauser & Carvalho 2008; Hutchings et al. 2010, Hutchings & Reynolds 2004, Therkildsen et al. 2010). Under this model N_e of marine populations is predicted to be extremely large and would behave like an idealized population where drift is negligible, i.e. N_e is effectively infinity. Comparison of the genetic diversity of freshwater, anadromous and marine fish shows that marine species have higher genetic diversity than freshwater fish, with anadromous species falling in between them (DeWoody & Avise 2000, Ward et al. 1994). Generally, this is attributed to differences in the level of population structure resulting from differences in habitat and its effect on N_e . Freshwater species inhabit lakes, rivers and streams, which are thought to harbor relatively small, genetically isolated populations with low N_e when compared to marine populations. Freshwater fish are therefore predicted to lose genetic diversity more quickly than marine fish, which is consistent with empirical observations.

There is an increasing amount of evidence that marine fish may have lower N_e than is traditionally thought and may sometimes be vulnerable to genetic drift, bottlenecks and inbreeding. First, marine fish populations are commonly more structured geographically than was thought given the ability of larval and adult stages to move long distances (see Hauser & Carvalho 2008 for review). This is attributable to several factors, including philopatry, i.e., individuals homeing back to their natal to reproduce, and larval retention being more common than long distance dispersal. In addition, life history characteristics and environmental

stochasticity generally result in N_e being substantially smaller than the total population of adults (the “census population size” [N_c]). Frankham (1996) found the N_e/N_c ratio in wild populations to be approximately 0.1, a similar result to Palstra & Ruzzante (2008). However, in species with a type III survivorship N_e can be 3-5 orders of magnitude smaller than N_c (Hauser et al 2002, Hoarau et al. 2005, Turner et al. 2002). Type III survivorship is common in marine fish, hence N_e is often likely to be dramatically smaller than N_c (Hauser et al. 2002, Hoarau et al. 2005, Turner et al. 2006). When coupled with recent, severe population declines in many marine fish due to overexploitation and/or habitat loss and degradation (Reed 2004), N_e of many of these species may be at levels low enough for drift, bottlenecks and inbreeding to negatively affect their genetic diversity. Loss of genetic diversity due to genetic drift and inbreeding can limit adaptive potential and their ability to resist diseases and environmental fluctuations (Nei 1975, Willi et al. 2006). Considering that marine fish are now living in an era of relatively rapid climate change, their ability to adapt could be an important factor determining their long-term persistence.

There are growing concerns that the N_e of marine fish is not typically functionally equivalent to an infinite population and they may therefore be vulnerable to processes with the potential to erode their genetic diversity. In light of this, I reviewed the current literature on N_e estimation and evidence of genetic bottlenecks and inbreeding in marine fish. I first characterized when these studies were published and what taxa and regions are currently the best represented in the literature. I then summarized what we know about the magnitude of N_e in marine fish that have been published to date, with specific interest in how commonly estimates yield an infinite value expected for very large populations. Lastly, I aimed to compile evidence of genetic

bottleneck and inbreeding signatures occurring in marine fish populations, which can both be symptoms of small N_e in recent or contemporary generations.

Methods

I will first review the sampling and analytical methods that have been used to estimate N_e , detect bottlenecks and assess inbreeding levels in wild populations. I will then describe my literature search to locate peer-reviewed publications in which these methods were applied to marine fish.

Estimation of effective population size

For populations with a known N_c it is possible to use demographic parameters, including sex ratio and reproductive success of breeding individuals, to determine N_e (Caballero 1994, Engen et al. 2005). This is of limited practical use, however, because N_c is typically unknown in free-living animal populations and especially for those that live in the marine environment. Genetic estimation of N_e is increasingly feasible given recent analytical advances and the increasing availability of species-specific microsatellite markers, although these methods are still underused in studies of certain taxa (see Hare et al. 2011, Leberg 2005, Luikart et al. 2010, Waples & Do 2009 for review). These methods are broadly divided into “temporal estimators” and “single sample estimators”. Temporal estimators require samples taken several generations apart and are based on relating the standardized variance of allele frequencies across several generations to N_e (Nei & Tajima 1981). There is a wide range of temporal methods that have been implemented in various programs (reviewed in Leberg et al. 2005, Luikart et al. 2010), ranging from moment estimators (Jorde & Ryman 1995, 2007, Peel et al. 2004, Waples 1989), pseudo-likelihood methods (Wang 2001, Wang & Whitlock 2003), coalescent Bayesian methods (Beaumont et al. 2002, Berthier et al. 2002) and approximate Bayesian computation (Cornuet et

al. 2008). The drawback to all of these methods is that they require multiple sample sets spaced several generations apart, which can be challenging to obtain for marine fish.

Recent analytical advances enable genetic estimation of N_e using a single sample of individuals from a population. The three most commonly used ones are the linkage disequilibrium method (“LD”) (Waples 2006, Waples & Do 2008, 2009), Bayesian approximation (“BA”) (Tallmon et al. 2008) and the heterozygote excess method (“HE”) (Pudovkin et al. 1996). In all cases it is assumed that the population is randomly sampled and the microsatellite loci are unlinked and statistically independent. The study population is also assumed to be an idealized Fisher-Wright population in which genetic drift is the only factor affecting genetic diversity, i.e., there is no selection, migration, mutation. Bayesian approximation computes N_e over several generations in the past using summary statistics, e.g. variance in allele length and heterozygosity, number of alleles per locus, expected heterozygosity (Beaumont et al. 2002, Tallmon et al. 2008). The HE method is based on the statistical differences in allele frequencies between males and females generated by binomial sampling effects occurring in very small populations resulting in the offspring being more heterozygous than the parental generation. This method has very low precision and is only applicable for very small populations (< 30 breeding adults) and is therefore unlikely to be useful for marine fish (Leberg et al. 2005). The LD method is based on the small amount of linkage of alleles that occurs during recombination due to sampling error in very small populations. The Key assumption for the LD method is that this linkage disequilibrium signal is driven solely by genetic drift and is related to N_e . Because it requires small samples size of only 30-50 individuals and a minimum of 10 loci, this method has gained the most traction for estimating N_e of wild populations and has been extensively reviewed and tested using simulations (Palstra &

Fraser 2012, Waples & Do 2009). In species with overlapping generations, i.e., an age structured population, it is difficult to randomly sample and estimate N_e for a single generation.

Simulations have shown that a random sample of mature adults provides the closest estimate of N_e for the generation they are a part of. In contrast, a random sample of a single cohort or series of cohorts estimates a parameter called the effective number of breeders (N_b). The effective number of breeders approximates the number of parents that produced the sampled cohort(s).

The harmonic mean of N_b for each cohort making up a generation equals N_e for that generation (Waples & Do 2009). The usefulness of genetic N_e and N_b estimators have been extensively reviewed (Leberg et al. 2005, Luikart et al. 2010) and have even been discussed in the context of how they may be applied to marine organisms specifically (Hare et al. 2011).

Assessing genetic bottlenecks

Recent progress in statistical methods and the availability of molecular markers allow for bottleneck tests using a single sample e.g. M-ratio test (Garza & Williamson 2001), the heterozygote excess test (Cornuet & Luikart 1996, Piry et al. 1999) and the mode shift test (Luikart et al. 1998), all of which detect the genetic signature of declines in N_e . The Key assumption for all three methods is that during a bottleneck rare alleles are lost more quickly than common ones. The heterozygote method is based on the fact that in very small population statistical differences will occur in the allele frequencies between males and females resulting in the offspring being more heterozygous than the parental generation. This method will most effectively detect a recent, very severe bottleneck. If low N_e persists the signal can be confounded by inbreeding which results in an excess of homozygotes (Cornuet & Luikart 1996, Piry et al. 1999). The mode shift test is a more conservative estimator with a more limited power to detect bottlenecks. For this method allele frequency classes are established. For most

microsatellite loci the lowest frequency class is the mode allele frequency. Shifts in allele frequency distribution are still detectable for several generations after a bottleneck, in which rare alleles are preferentially lost, the mode shifts from the lowest to a moderate frequency class (Luikart et al. 1998, Piry et al. 1999). The M-ratio test assumes that during a bottleneck the number of randomly lost alleles will increase more quickly than the range of their sizes decreases. Further, it assumes that under the stepwise mutation model in a robust population most allelic states should be occupied. With rare alleles being lost during a bottleneck the number of unoccupied states will increase, whereas the total size range will only change if the smallest and largest alleles are lost. The proportion of unoccupied potential allelic states is measured by $m=k/r$, with k = the number of observed allelic states in a sample and r = the number (range) of potential allelic states (Garza & Williamson 2001). Assessment of these methods to determine adequate sample size and number of loci necessary to reliably test for bottlenecks is ongoing (Peery et al. 2012).

Assessing inbreeding

Inbreeding is defined in a population genetic context to mean a deviation from random mating whereby individuals mate with related individuals more commonly than expected by chance (Jacquard 1975). Inbreeding is predicted to cause deviations from Hardy-Weinberg Equilibrium (HWE), specifically a deficit of heterozygous individuals relative to expectations based on random mating (Wright 1922). Deviations from HWE are most commonly measured using the inbreeding coefficient (F_{IS}) for the population, which is defined as the probability that two homologous alleles are identical by descent, i.e. that they are identical copies of a single common ancestral allele (Wright 1922, 1965). These HWE deviations can also be caused by technical (e.g., null alleles) or sampling artifacts (e.g., Wahlund effects) and these need to be

thoroughly investigated and ruled out before attributing high F_{IS} to inbreeding (Capuis & Estoup 2007, Castric et al. 2002, Dhamarajan et al. 2012, Lemer et al. 2011, Wahlund 1928). There are also several ways to measure inbreeding within individuals, including multi-locus heterozygosity (MLH) and internal homozygosity levels (HL). While MLH indicates the proportion of individuals within a sample heterozygous at 1, 2, ... loci (Castric et al. 2002, Slate et al. 2004), HL indicates the proportion of loci and individual is homozygous for (Aparicio et al. 2006, Frasier 2008). Amos et al. (2001) suggested calculating the internal relatedness (IR) of sampled individuals, which determines the level of relatedness of the parents of an individual. Similarly, to MHL and HL, IR determines the proportion of genotyped loci of an individual that are homozygous but weights alleles sharing according to how rare it is in the population. IR ranges from 0 to 1, with 0 indicating individuals that are completely outbred and 1 that they are clones. This method has been implemented in the program STORM (Frasier 2008).

Literature review

A literature search was performed using the Key words “effective population size”, “bottleneck”, “inbreeding” in combination with “fish” using major databases (Biological Abstracts, BioOne, PubMed, Web of Science and GoogleScholar to find publications in online preview before being included in print editions of their respective journals) through May 1st, 2013. I also searched citation records of Key methods papers for Ne estimation, bottleneck detection and inbreeding detection (Frasier 2008, Garza & Williamson 2001, Piry et al. 1999, Pudovkin et al. 1996, Tallmon et al. 2008, Waples 1989, 2006, Waples & Do 2008) and separated out studies focused on marine fish. To limit the scope of this review salmonids, which are anadromous and have been reviewed elsewhere (Waples 1990a; Waples 1990b), were excluded. After relevant studies were identified, the trophic level and the status of commercial

fisheries and aquaculture for each species were extracted from www.fishbase.org. Furthermore the IUCN (International Union for Conservation of Nature) red list status and the FAO (Food and Agricultural Organization) region (<http://www.fao.org/fishery/area/search/en>) of the study population were obtained using the IUCN online database (<http://www.iucnredlist.org/>) and fishbase (www.fishbase.org). I included in the review any peer reviewed study that used microsatellite-based analytical methods to (1) estimate N_e , (2) attempt to detect a recent bottleneck, (3) assess inbreeding or (4) perform any combination of (1)-(3) in at least one wild marine fish population. Due to inconsistent terminology used to describe the type of N_e estimated (e.g., contemporary vs. historic N_e , N_b) I grouped all N_e estimates together for analysis. Not all studies provided 95% confidence intervals (CI) for N_e point estimates, but when these were available I noted when the upper CI was infinity. Conversely, not all studies provided point estimates; some studies only cite an estimated range. Studies attempting to detect recent genetic bottlenecks in marine fish populations were included if they used either temporal or single sample methods. Several studies used multiple methods to assess the occurrence of bottlenecks and the results were not always consistent within one study; in these cases we followed the conclusions of the authors as to whether or not there was sufficient evidence documenting a bottleneck. To assess the prevalence of inbreeding in marine fishes we surveyed microsatellite studies of marine fishes and extracted those with deviations from Hardy-Weinberg equilibrium (i.e., heterozygote deficits) in which the authors also employed methods to rule out technical or sampling artifacts and concluded that inbreeding was responsible for this pattern. For N_e estimation, bottleneck detection and inbreeding assessment I also included results from the four species included in this volume (i.e., Key silversides, *Menidia conchorum* [Chapter 2],

winter flounder, *Pleuronectes americanus* [Chapter 3], Atlantic sturgeon, *Acipenser oxyrinchus* [Chapter 4] and white shark *Carcharodon carcharias* [Chapter 5]).

Results and Discussion

Ne estimates in marine fish

After the first study estimating N_e in a marine fish population was published in 1999 the publication rate has accelerated with almost 80% of studies appearing in the last 5 years (Figure 1A). A total of 37 studies were identified assessing N_e in 86 populations of marine fishes of 37 species (Table 1). Approximately 80% of marine fish populations for which N_e has been estimated are classified as “commercial” or “highly commercial” while only 7% of the species studied were classified as “not commercial” (Figure 2A). This suggests that fisheries management needs are a primary driver of the collection of genetic data in marine fish. A large proportion of the study species (45-50%) are classified as not evaluated or data deficient by the IUCN Redlist and almost 20% are “endangered” or “critically endangered” (Figure 2B). The latter indicates that conservation concerns are also a Key motivator of population genetic research on marine fish. There is a strong skew towards populations in the Northeast Atlantic with about 25-35% of the study populations located there, followed by 10-15% in the Northwest Atlantic and only a very small proportion located in the Pacific (Figure 2C). This regional pattern likely stems from the proximity of study populations to genetic research capacity that primarily resides in Europe and the U.S.A. Nearly 40% of the study species belong to the order of Perciformes, followed by just over 10% in the order of Pleuronectiformes (Figure 2D).

Effective population size estimates ranged from approximately 10 individuals to $> 10^6$ individuals (Figure 3A). In only 16% of the studied populations the reported point estimate of N_e was infinity (Figure 3E) and in 25% of the studies with a finite point estimate the upper C.I.

boundary was not distinguishable from infinity. Finite point estimates were reported for 84% of the study populations and for 75% of those populations there were finite boundaries. The fact that relatively few of these populations exhibited N_e of infinity indicates that they are potentially affected by genetic drift. In general, theoretical calculations suggest that having a minimum N_e between 5,000 and 12,500 is required to maintain long term genetic diversity (Franklin & Frankham 1998). The 50/500 rule (Franklin & Frankham 1998) proposes that populations with $N_e < 50$ are susceptible to inbreeding while $N_e > 500$ must be maintained to avoid the loss of adaptive potential. Other theoretical studies place that number higher and state that a minimum $N_e = 500-5000$ is necessary to maintain the evolutionary and adaptive potential (Lynch & Lande 1998). Overall, for 10% of the study populations $N_e < 50$, 21% had point estimates between 50 and 500 and N_e ranged from 500-5000 individuals for 31%. According to the studies we recovered only 21% of the populations had sufficient N_e to maintain long-term stability (Figure 3B). For 16 of the assessed studies it was possible to compare N_e and N_c . The effective population size was determined to be 3-5 orders of magnitude smaller for broadcast spawners characterized by a type III survivorship curve, i.e. a large number of offspring with high juvenile mortality and a high variation in reproductive success (Hauser et al. 2002, Hoarau et al. 2005, Turner et al. 2002). For long-lived species such as sharks and sturgeon the ratio was found to be considerably higher, ranging from 0.4 – 0.5 (Duong et al. 2013, Portnoy et al. 2009).

The studies of N_e in marine fish to date support the emerging paradigm that certain marine fish populations are smaller (both in terms of N_c and N_e) and more isolated than traditionally thought. The studies we reviewed are likely to be biased towards those populations that have low to moderate N_e , however. First, it is clear that commercially exploited and endangered species made up a high proportion of the study species, both of which by definition

have experienced recent declines in N_e (Figure 3). It is also clear that majority of these studies were not primarily conceived to assess N_e ; instead estimation of N_e was a side product of studies focused on assessing population genetic structure. As a result, I suspect a bias towards reporting “positive” results where a non-infinite point estimate was obtained. Other researchers may have estimated N_e to be infinity and not reported it in their broader population genetic study because they felt it was not especially insightful. In order to overcome this bias in the future, I suggest that all microsatellite studies of marine fish should estimate and report N_e . This will enable us to determine whether for most species N_e really is functionally equivalent to infinity and will also allow us to conclusively determine what the major drivers of low N_e in marine fish are.

Despite the skew towards endangered and heavily exploited species and likely publication bias, I found bound estimates of N_e have been obtained for a wide variety of marine fish. The magnitude of N_e is often surprisingly low and only occasionally was N_e estimated to be infinity or have an unbound upper CI, indicating that drift is a threat to the genetic diversity of at least some marine fish populations. One issue that is apparent with many of the existing studies is that it is not made clear what the estimate of N_e actually represents (Palstra & Fraser 2012, Waples & Do 2009). The effective number of breeders, N_b , for any period less than the generation time of the species is likely to be markedly smaller than the N_e for the generation. Many researchers may be inadvertently estimating a parameter closer to N_b than N_e by virtue of non-random sampling of the generation (Palstra & Fraser 2012, Waples 2005). For example, if a certain age class is overrepresented in a sample then it is likely that N_e estimates are actually closer to the N_b that produced this group of individuals. On the other hand, marine fish often exist as a series of metapopulations, whereby a number of largely demographically independent subpopulations are connected by small amount of migration between them. Simulations have

shown that even a small amount of migration into a local population means that N_e estimated from a sample of individuals from the local population most closely approximates N_e for the whole metapopulation (Charlesworth 2009, Gilpin 1991, Wang & Whitlock 2003). In order to correctly interpret this N_e it is therefore essential to understand the population structure. Given these issues, I recommend that researchers fully disclose the size and/or age distribution of each population sample and make an explicit statement (which can be reviewed for accuracy) about whether they are estimating N_e or N_b , and, for the latter, define the period of time it refers to. In addition, all attempts should be made to define the spatial boundaries of the population for which N_b or N_e is being estimated. While N_e is an important parameter to estimate from the perspective of assessing the risk of genetic diversity loss to drift and long-term evolutionary potential, resource managers should be made aware that robust, transparent estimates of N_b for specific cohorts or groups of cohorts provide an estimate of the number of breeding adults that produced the cohorts (Palstra & Fraser 2012, Waples 2005). Although it is likely skewed towards the limiting sex, enumerating the number of breeding individuals is frequently extremely useful for management and conservation. My review suggests that it will be possible to estimate and track this parameter over time in a wide variety of endangered and exploited marine fish populations using genetic methods.

Genetic evidence of recent bottlenecks in marine fish

Genetic bottlenecks may occur when N_e is temporarily but drastically reduced. Given the number of studies showing low N_e in marine fish, I expected that to find evidence of genetic bottlenecks in at least some of them. Twenty-seven studies were identified that attempted to detect a genetic bottleneck in 33 populations of 25 species of marine fish (Table 1). Similar to N_e estimation, approximately half of these studies were published in the last 2-3 years (Figure 1B).

Similar to marine populations for which N_e was estimated, 80% of the populations tested for evidence of bottlenecks are classified as “commercial” or “highly commercial” and only 9% were not commercial (Figure 6-4A). 40% of the study species are categorized as “not evaluated” by the IUCN, while 11% were categorized as “critically endangered” or “endangered” (Figure 4B). Further, the majority of study populations are found in the Northeast (27%) and Northwest Atlantic (15%) and only 10% located in the Pacific (Figure 6-4C). Again Pleuronectiformes and Perciformes are the most strongly represented orders, comprising 15% each. The remaining 70% are comprised of fishes belong to twelve different orders (Figure 6-4D). Bottlenecks were detected in approximately half of the populations tested (Figure 6-5A). In 61% of the studies testing for bottlenecks N_e was also estimated (Figure 6-5C), which is not surprising given the link between them.

While estimating N_b and generational N_e can give insight in to the current status of potential loss of genetic diversity, testing for bottlenecks enables a glimpse of the recent demographic history of a population. Genetic bottlenecks are symptoms of a temporary reduction in N_e and can result in low population genetic diversity even despite full or partial recovery of census population size. Overall, bottlenecks were detected in less than half of the populations assessed and nearly all bottlenecks were detected using the M-ratio test (Figure 6-5B). This may reflect the fact that all three of the commonly employed bottleneck tests have been shown by simulations and empirical assessments of known bottlenecked populations to have low statistical power. Type II error, i.e., failing to detect a real bottleneck, appears to be a common issue when using these tests (Peery et al. 2012). In addition, it is possible that demographic bottlenecks do not always cause genetic bottlenecks. For example, Chapman et al. (2011) found no genetic evidence of a bottleneck in the Florida population of small-tooth sawfish, *Pristis pectinata*

despite this species having experienced a severe decline in abundance and geographic range. Simulated projections of genetic diversity in *P. pectinata* showed that life history characteristics such as long generation time could act as a buffer to loss of genetic diversity (Chapman et al. 2011; DeHaan et al. 2006, Lippe et al. 2006). Regardless of these issues, the increasing number of reports of genetic bottlenecks being detected in marine fish is cause for concern given the potentially strong effect on genetic diversity (Nei 1975).

Genetic evidence of inbreeding in marine fish

Only 6 studies reported evidence for inbreeding (Table 6-1, Figure 6-6): in two flatfish species, European plaice, *Pleuronectes platessa*, (Hoarau et al. 2005), and winter flounder, *Pseudopleuronectes platessa*, (O'Leary et al. 2013b, Chapter 3) as well as two shark species, blacktip reef sharks, *Carcharhinus melanopterus*, (Mourier & Planes 2013) and white sharks, *Carcharhinus carcharias*, (Chapter 5), Atlantic sturgeon, *Acipenser oxyrinchus* (Chapter 4) and *Menidia conchorum* (Chapter 2). Five of these studies used IR in addition to the inbreeding coefficient to determine incidences of closely related parents breeding. For three of these species mean IR was 0.2 or higher, indicating breeding on the level of half-siblings or higher (Figure 6-6). For the other two species while mean IR were 0.02 – 0.03 (Figure 6-6), approximately 10% of individuals in the sample population exhibited IR-levels higher than 0.2. When the first incidence of inbreeding in a marine fish was discovered in European plaice (Hoarau et al. 2005), the authors attributed it to a greatly reduced population size, high fidelity to spawning grounds and homing behavior. Similarly, winter flounder displays fine scale population structure in the bays of Long Island in addition to extremely low N_b , 60 -150 individuals per bay. Both of these factors could contribute to a recent study (O'Leary et al. 2013b) discovering that the parents of sampled individuals were on average related on the level of half-siblings. In the most extreme

case, *Menidia conchorum* inhabits hypersaline pools in the Florida Keys which are tidally restricted and gene flow is almost non-existent. The extremely fragmented habitat has led to a prevalence of inbreeding and levels of genetic diversity and fluctuation thereof that closely resemble freshwater fish. The recent evidence of inbreeding in long-lived sharks and sturgeon is even more unexpected considering they are highly mobile species capable of long-distance migration. Yet, these species display population structure, and increasing local fishing pressure can result in populations becoming fragmented and isolated which would make them susceptible to both low N_e and inbreeding. A recent study of white sharks found N_b in the North Atlantic to be less than 50 individuals and the mean level of parental relatedness of sampled individuals was equivalent of half-siblings mating (Chapter 4). Mourier & Planes (2013) found that 8% of sampled black-tip reef sharks' parents were related on the level of half-siblings, and 38% on the level of cousins. This species is highly residential around oceanic islands and it is therefore likely that individuals could encounter relatives during the mating season. Similarly, Atlantic sturgeon display strong population structure as they home to their natal rivers to spawn, and a recent study found that 10-12% of individuals in three of these natal rivers had parents related at the level of half-siblings or higher (Chapter 4).

In contrast to the widely held belief that inbreeding does not occur in marine fishes, a literature revealed six studies citing evidence of inbreeding, indicating that it could be more common than reflected in studies published to date. On the other hand, heterozygote deficiencies, a potential indicator of inbreeding, are commonly reported in microsatellite studies of marine fish (e.g. Florin & Hoglund 2008, Karlsson & Mork 2005, McCairns et al. 2012, Norouzi et al. 2012, Wang et al. 2007, Zarraonaindia et al. 2009). In most cases these are attributed to technical (null alleles) or sampling artifacts (Wahlund effect, Wahlund 1928) and inbreeding is not

considered as an alternative hypotheses. In light of recent evidence for the occurrence of low N_e and inbreeding in some marine fish I suggest more rigorous testing of inbreeding as a hypothesis to explain HWE deviations is needed (Castric et al. 2002, Dhamarajan et al. 2012, O'Leary et al. 2013b). An excess of homozygotes can have several explanations (Hardy 1908, Weinberg 1908) and each possible explanation should be considered equally when such a discovery is made. Null alleles, non-amplifying alleles due to point mutations in the flanking region of microsatellites, can result in false homozygotes. It is possible to test for null alleles independently of the HWE-based method by lowering annealing temperatures during PCR and redesigning primers. A frequently overlooked method is to exhaustively amplify all individuals in the sample. If all individuals amplify at all loci then high frequency null alleles are an impossibility because some individuals should be null homozygotes and chronically fail at one locus while amplifying at all others. The Wahlund effect is an excess of homozygotes caused by inadvertent mixing of multiple differentiated gene pools into a single sample (Wahlund 1928), and in its most extreme form occurs when closely related individuals are prevalent in the sample. Wahlund effects can be avoided by testing for population structure within the sample using Bayesian clustering, while family structure can be revealed by testing for pairwise relatedness of sampled individuals (Castric et al. 2002, Dhamarajan et al. 2012). I suggest that studies that find HWE deviations within marine fish populations should routinely determine whether they result from null alleles or Wahlund effect using these procedures and therefore identify situations where inbreeding is the most likely explanation.

Conclusions

Marine fish populations were once famously described as being “inexhaustible” (Huxley 1883) and although that idea has long since been discredited it has only been recently that we

have recognized that processes such as philopatry, retention of young in the natal area and skewed reproductive success make their population biology very similar to most other vertebrates. This review shows that genetic drift is a potential threat to the genetic diversity of a wide range of marine fish and that signatures of small N_e , bottlenecks and inbreeding, are now starting to be documented in marine fish populations all over the globe. Although the existing literature is likely biased towards populations with small N_e because most studies focus on endangered and exploited species and positive results, I suggest that if researchers begin reporting these estimates as a matter of routine we will obtain a fuller understanding of what factors lead to low N_e in marine fish. Future estimates of N_e should also be explicit about what parameter is estimated and resource managers should be made aware that estimates of N_b producing a given cohort or series of cohorts is a possibility in marine fish and has direct and tangible application in the management of exploited and endangered species. I also suggest that bottleneck testing and systematic assessment of the true cause of Hardy-Weinberg deviations will further illuminate the changing genetic architecture of marine fish populations. Overall, my review shows that evidence is rapidly accumulating that marine fish ranging from highly migratory oceanic apex predators like Atlantic bluefin tuna (*Thunnus thynnus*) to demersal, estuarine species like winter flounder (*Pleuronectes americanus*) that were once extremely abundant have reached low enough levels in some places and are subject to the same processes that erode genetic diversity in freshwater and terrestrial endangered species.

Figure 6-1: Annual trends of studies determining effective population size (N_e) and bottlenecks based on genetic data. (A) Number of studies assessing N_e for marine fish populations from 1999 to present. (B) Number of studies testing for evidence of genetic bottlenecks within marine fish populations from 2004 to present.

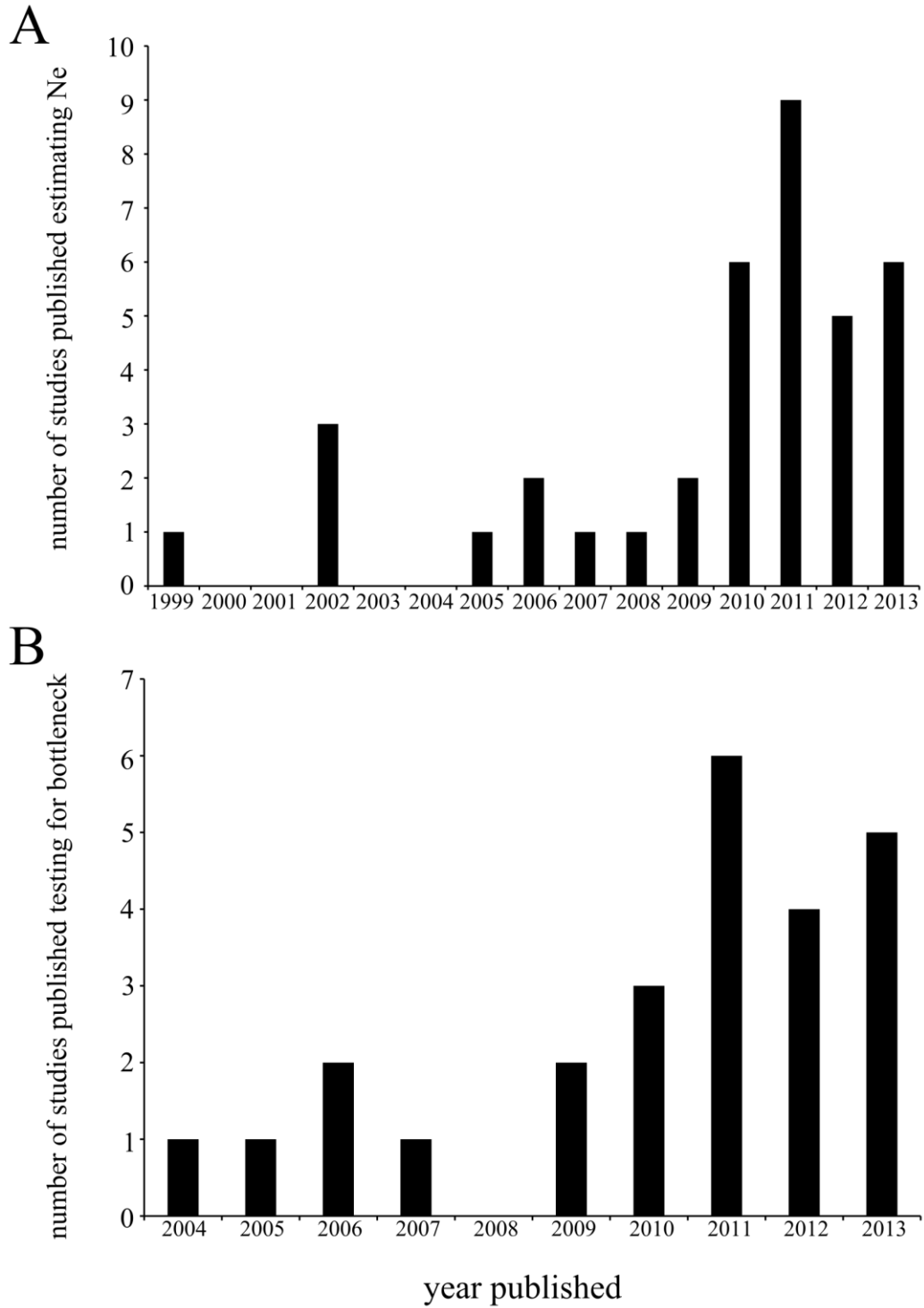


Figure 6-2: Categorization of study populations with effective population size (N_e) estimates according to commercial value, conservation status, geographic provenance and taxonomy. (A) Proportion of study populations supporting commercial fisheries and aquaculture. (B) Proportion of study populations listed on the IUCN Redlist. (C) Geographic provenance of study populations by FAO region. (D) Represented orders of fishes.

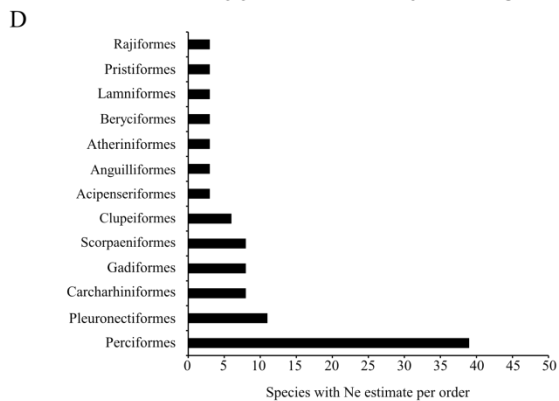
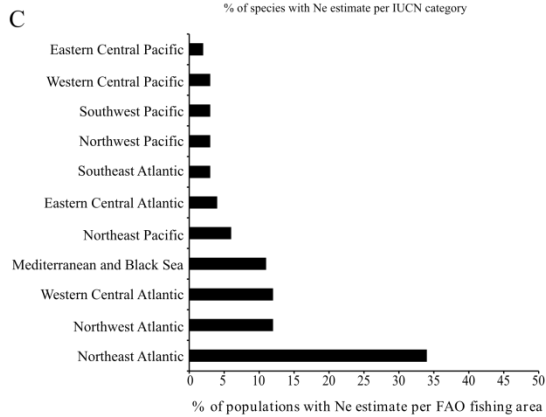
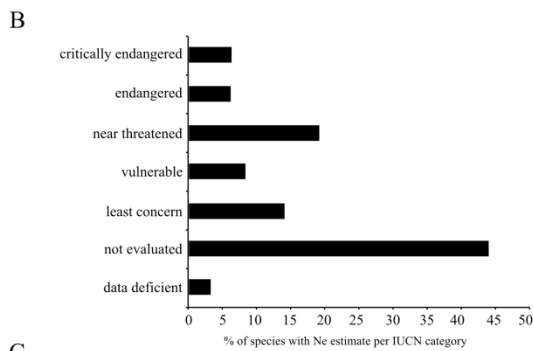
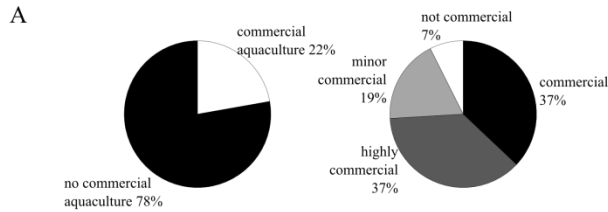


Figure 6-3: Effective population size (N_e) estimates. N_e estimates of all study populations using the linkage disequilibrium method. For studies for which only range estimates were cited the middle of that range was included. (A) Commercial value of study population by N_e estimate (category – color) (B) Conservation status of N_e estimates (category – color). (C) N_e estimates by geographic provenance (category – color). (D) Trophic level of analyzed populations by N_e estimates. (E) Frequency distribution of N_e estimates in size categories indicating critical population sizes below which inbreeding and loss of adaptive potential is of concern and populations large enough to maintain adequate genetic diversity long-term.

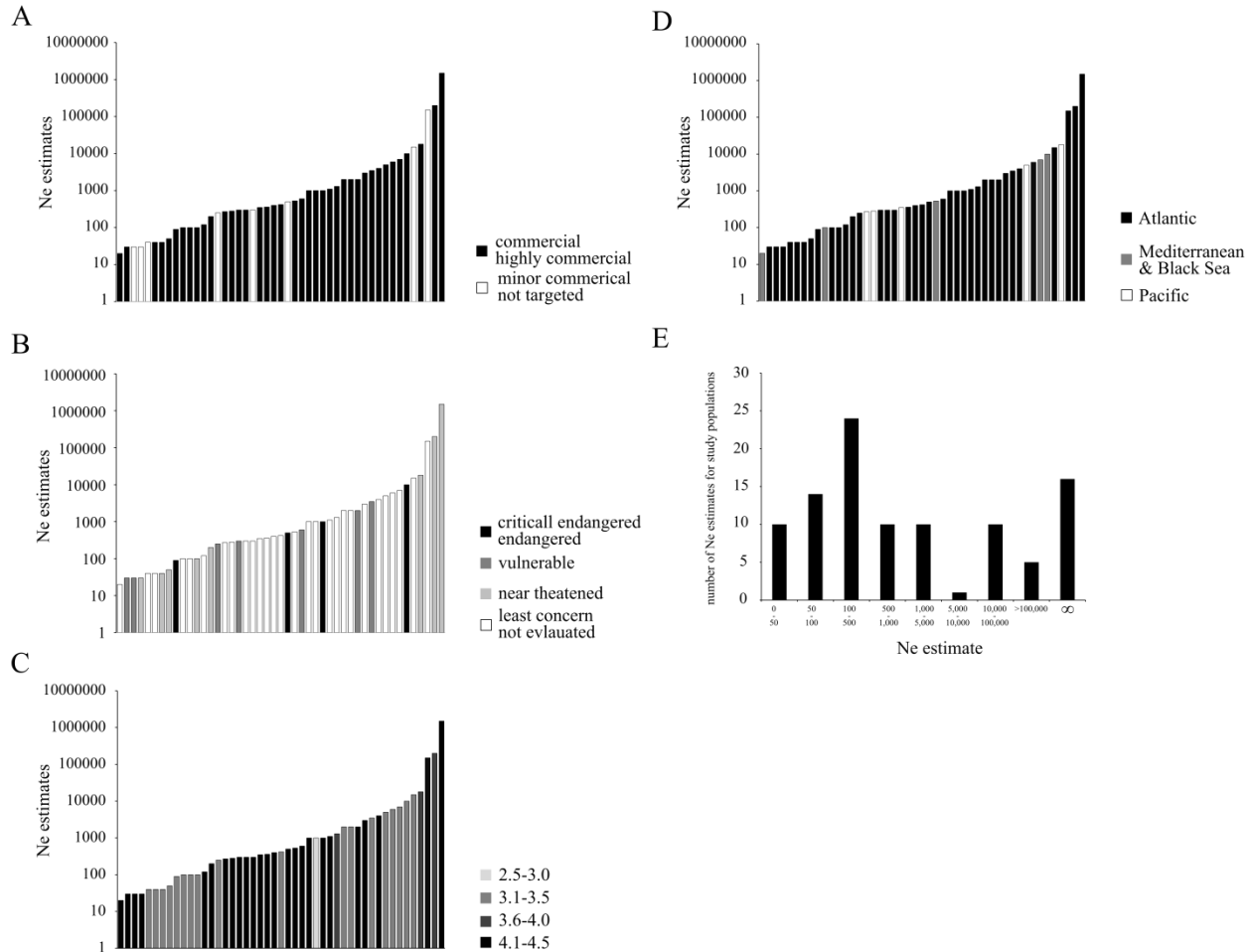


Figure 6-4: Categorization of study populations tested for genetic evidence of bottlenecks according to commercial value, conservation status, geographic provenance and taxonomy. (A) Proportion of study populations supporting commercial fisheries and aquaculture. (B) Proportion of study populations listed on the IUCN Redlist. (C) Geographic provenance of study populations by FAO region. (D) Represented orders of fishes.

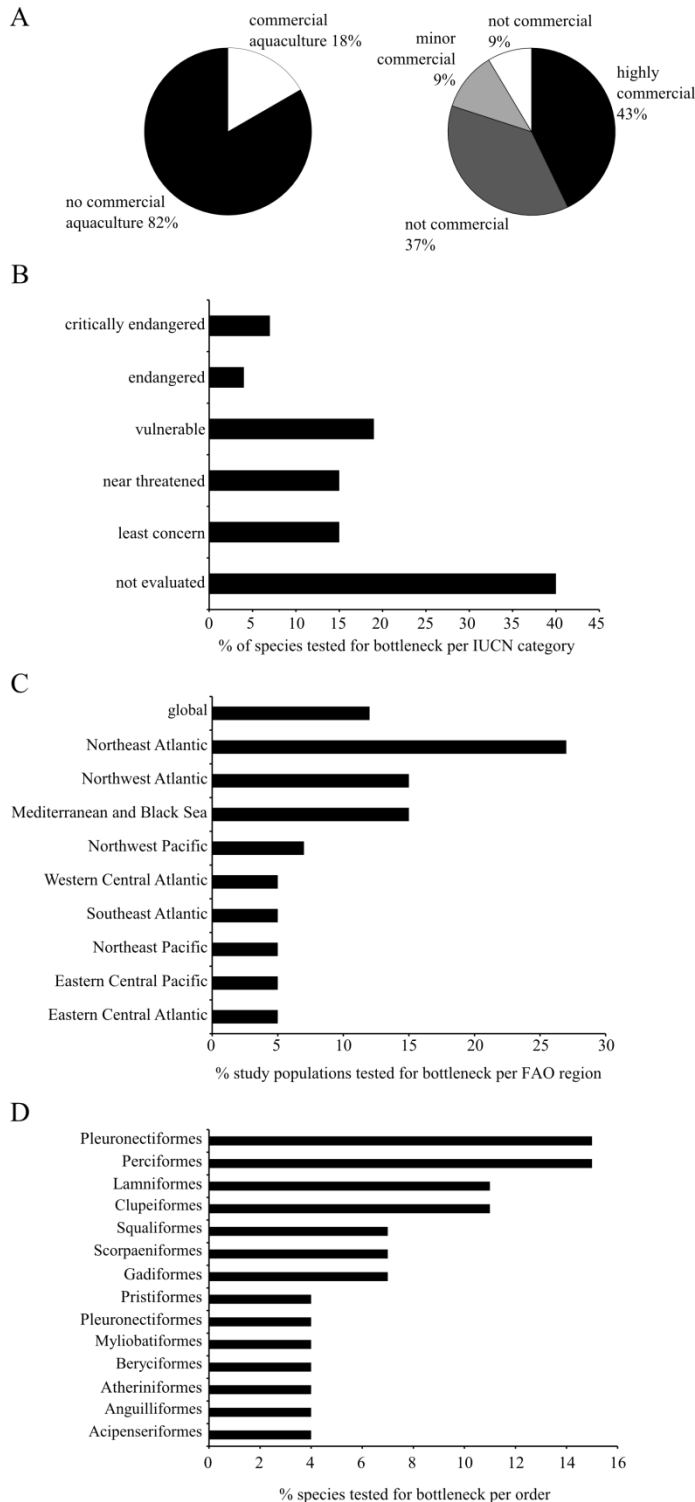


Figure 6-5: Results of bottleneck tests. (A) Proportion of study populations for which bottleneck was detected using genetic methods. (B) Detected bottlenecks by method (C) Proportion of tested populations for which N_e was also evaluated.

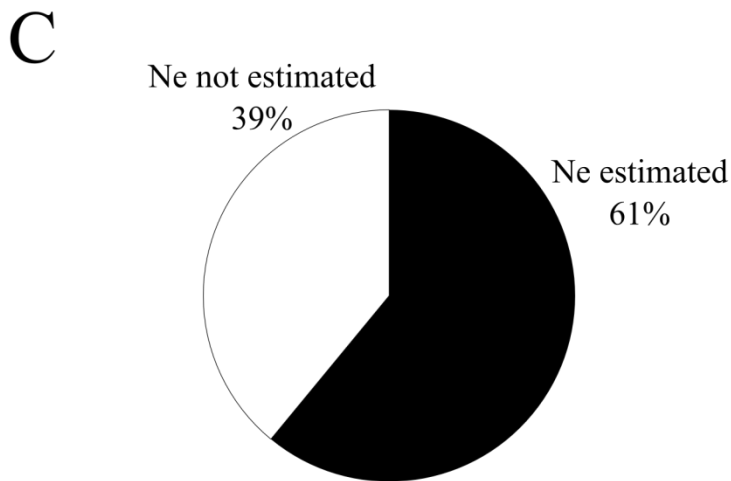
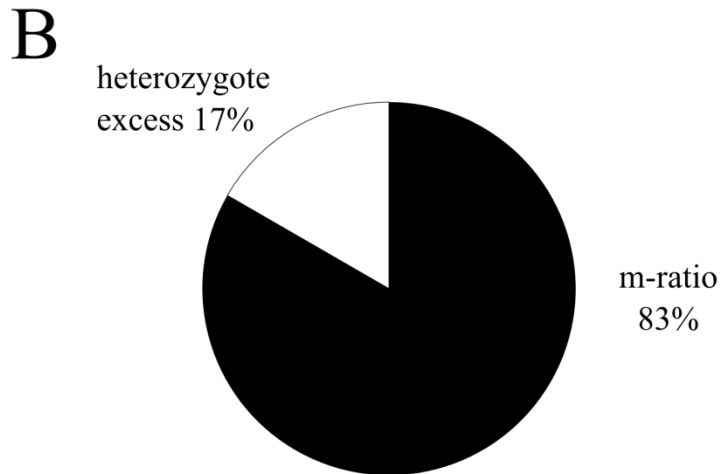
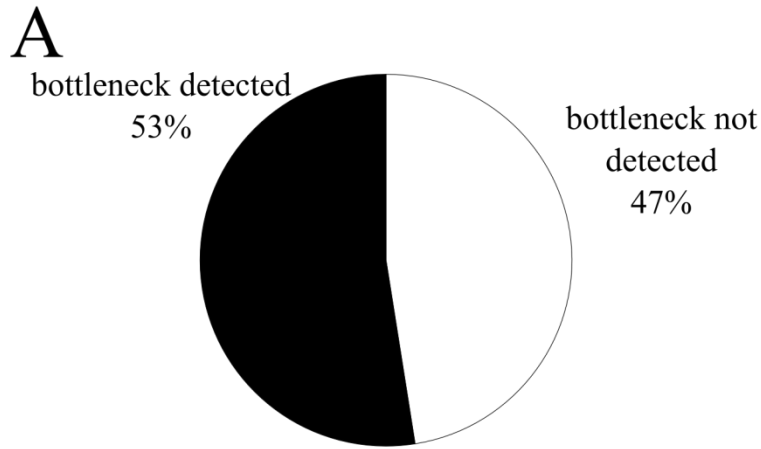
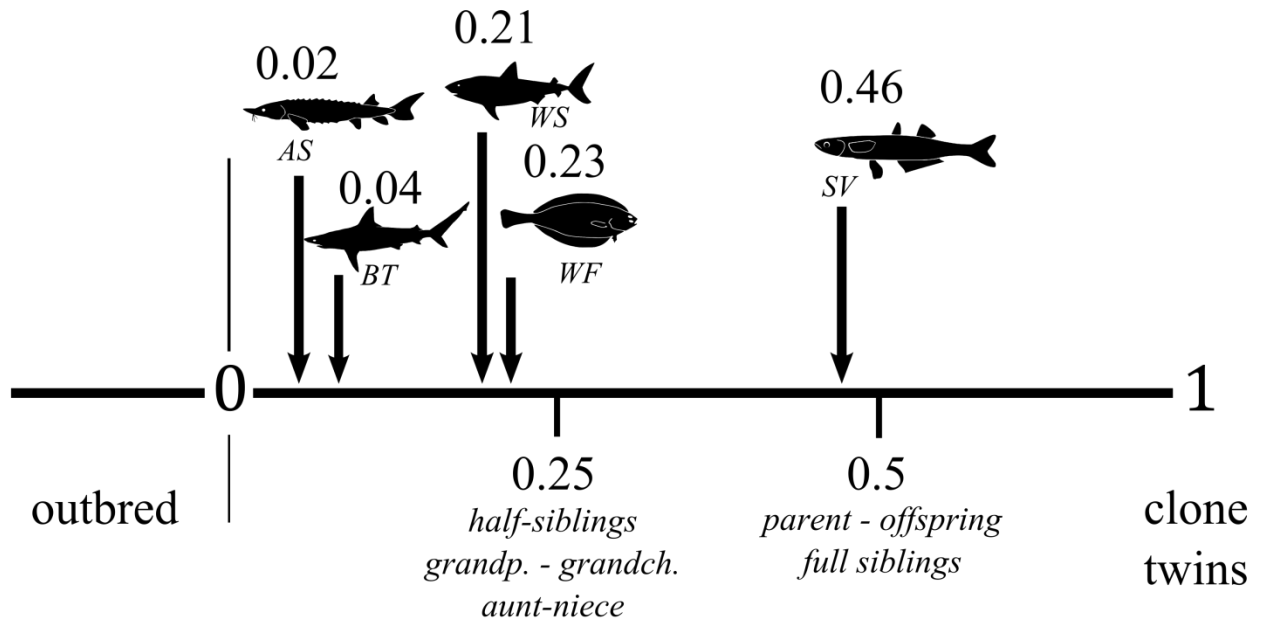


Figure 6-6: Mean level of internal relatedness (IR) for marine fish populations in which inbreeding was detected. Inbreeding was detected in Atlantic sturgeon (AS), blacktip reef sharks (BT), white sharks (WS), winter flounder (WF) and tidewater/Key silversides (SV).



Internal Relatedness (IR)

Table 6-1: Reviewed studies of marine fish populations for which effective population size (N_e) was estimated and tested for evidence of bottlenecks (BN) and inbreeding (IN). Open circles indicated populations that were tested for BN, closed circle indicates that bottleneck or inbreeding was detected.

Species	Population	N_e	ratio	BN	IN	Study
<i>Acipenser oxyrinchus</i>	James River, USA	45-100	LD	●	●	Chapter 4
	Delaware River, USA	75-180	LD	●	●	
	Hudson River, USA	170-230	LD	●	●	
<i>Anguilla anguilla</i>	Mediterranean	3×10^3 - 13×10^3	temp	○		Pujolar et al. 2011
<i>Antimora rostrata</i>	North Atlantic	15×10^3	BA	●		White et al. 2011
<i>Argyrosomus regius</i>	TurKey	20	LD			Haffray et al. 2012
	Mauritania	120	LD			
	Egypt	530*	LD			
	France	∞	LD			
	Portugal	∞	LD			
	Iberia	∞	LD			
<i>Carcharhinus leucas</i>	Northwest Atlantic	2×10^5 - 2.5×10^5	BA			Karl et al. 2011
	Southwest Atlantic	1.5×10^5 - 1.7×10^5	BA			
<i>Carcharhinus melanopterus</i>	French Polynesia	<i>n/a</i>			●	Mourier & Planes 2013
<i>Carcharhinus plumbeus</i>	Virginia, USA	1.5×10^3 - 3×10^3	LD	0.45 - 0.5		Portnoy et al. 2009
			temp			
	Delware, USA	3×10^3 - 4×10^3	LD	0.45 - 0.5		
			temp			
<i>Carcharodon carcharias</i>	Northwest Atlantic	30	LD	●	●	Chapter 5
	Southern Africa	250*	LD	●	●	
<i>Carcharias taurus</i>	global	<i>n/a</i>		○		Ahonen et al. 2009
<i>Centroscymnus coelolepis</i>	Eastern Atlantic	<i>n/a</i>		○		Verissimo et al. 2011
	Eastern Atlantic	<i>n/a</i>		○		
	Eastern Atlantic	<i>n/a</i>		○		
<i>Cephalopholis fulva</i>	St. Croix	300	LD			Portnoy et al. 2013
	West Puerto Rico	300	LD			
	St. Thomas	360	LD			
	East Puerto Rico	420	LD			
<i>Cetorhinus maximus</i>	global	<i>n/a</i>		●		Hoelzel et al. 2006
<i>Clupea harengus</i>	Sweden 1	400-4000*	temp			Larsson et al. 2010
			LD			
	Sweden 3	2000	temp			
			LD			
	Sweden 5	4000*	temp			
			LD			
	Sweden 2	∞	temp			
			LD			
	Sweden 4	∞	temp			
			LD			
<i>Clupea harengus</i>	North Sea	<i>n/a</i>		○		Mariani et al. 2005
<i>Epinephelus guttatus</i>	St. Croix	1100	LD			Portnoy et al. 2013
	West Puerto Rico	1300	LD			
	East Puerto Rico	3000	LD			
	St. Thomas	∞	LD			
<i>Epinephelus marginatus</i>	Mediterranean	2.5×10^3 - 3.5×10^3	HE			Shunter et al. 2011
			Co			
	Algeria	2.5×10^3 - 3×10^3	HE			
			Co			
	Senegal	3.5×10^3 - 4×10^3	HE			
		Co				

<i>Gadus morhua</i>	North Sea	70-120	temp	3.1x10 ⁻⁵			Hutchinson et al. 2003
	Canada	600-10 ⁴	temp LD BA				
<i>Hippoglossus stenolepis</i>	Baltic Sea	700-2x10 ³	temp		○		Poulsen et al. 2006
	North Sea	800-5x10 ⁵	temp		○		
	Aleutian Islands	270*	LD				Nielsen et al. 2010
	Bering Sea	280*	LD				
	Gulf of Alaska	350*	LD				
<i>Hoplostethus atlanticus</i>	Ireland	100 - 1000*	LD		○		Carlsson et al. 2011
			Co				
<i>Lutjanus campechanus</i>	Northeast Atlantic	2.2x10 ³	BA		○		White et al. 2009
	Namibia	8x10 ³	BA		○		
	Northeast Atlantic	2.2x10 ⁴	BA		○		
	Texa, USA	10 ³	temp				Saillant et al. 2006
<i>Menidia conchorum</i>	Alabama, USA	1.2x10 ³	temp				
	Louisiana, USA	>7.5x10 ⁴	temp				
<i>Pagrus auratus</i>	Florida Keys, USA	10 - 145	LD			● ●	Chapter 2
	Tasman Bay, NZ	100 - 200	HE,	10 ⁻⁵			Hauser et al. 2002
<i>Pleuronectes platessa</i>	Hauraki Gulf, NZ	100 - 10 ³	temp HE,	10 ⁻⁵			
			temp				
	Scotland	40	LD	10 ⁻⁵			Watts et al. 2009
	Scotland & Ireland	90	LD	10 ⁻⁵			
	Irish Sea	6x10 ³ - 7x10 ³ *	LD	10 ⁻⁵			
	Irish Sea	n/a				○	Watts et al. 2004
<i>Pristis pectinata</i>	Iceland	1.8x10 ³	temp	2x10 ⁻⁵		●	Hoarau et al. 2005
	North Sea	2x10 ⁴	temp	2x10 ⁻⁵		●	
<i>Psetta maxima</i>	Florida	200-1000	LD		○		Chapman et al. 2011
<i>Pseudopleuronectes americanus</i>	Baltic Sea	n/a			○		Florin & Hoglund 2007
<i>Raja clavata</i>	Long Island, USA	1000	LD			● ●	O'Leary et al. 2013b
<i>Rhomboplites aurorubens</i>	Irish Sea	150-800	temp	9x10 ⁻⁹ - 6x10 ⁻⁴			Chevolot et al. 2008
	US Atlantic	25 - 150x10 ³	Co	10 ⁻¹ - 10 ⁻³			Bagley et al. 1999
<i>Sardina pilchardus</i>	Gulf of Mexico		temp				
	Bay of Biscay	200-500	temp				Laurent et al. 2007
	Chioggia, Italy	50-1.5x10 ³	LD			●	Ruggeri et al. 2012
<i>Sciaenops ocellatus</i>			Co			●	
	US Atlantic coast	300-1000	temp				Chapman et al. 2002
	Gulf of Mexico	2.5x10 ³ - 3.5x10 ³	temp	10 ⁻³			Turner et al. 2002
<i>Scomberomorus commerson</i>			Co				
	Australia	>1.8x10 ⁴	LD				Macbeth et al. 2013
<i>Sebastes alutus</i>	Alaska	200 - >8x10 ³ *	LD		○		Palof et al. 2011
<i>Sebastes cramerii</i>	Eastern Pacific	9x10 ³	temp	10 ⁻³ - 10 ⁻⁴			Gomez-Uchida et al 2006
<i>Sebastes melanops</i>	California, USA	50-200	Ped		○		Lotterhos et al. 2012
	California, USA	50-200	Ped		○		
<i>Solea solea</i>	North Sea	2x10 ³ - 5x10 ³	temp	10 ⁻⁵ - 10 ⁻⁶			Cuveliers et al. 2011
			LD				
<i>Sparus aurata</i>	Northeast Atlantic	n/a			○		Guinand et al. 2013
	Ireland	40	LD			●	Coscia et al. 2011
	Spain	∞	LD			●	
	Portugal	∞	LD			●	
	France	∞	LD			●	
	Adriatic Sea	n/a				○	Segvic-Bubic et al. 2011
<i>Sprattus sprattus</i>	Atlantic	n/a			○		Limborg et al. 2012
	Atlantic	n/a			○		
<i>Squalus acanthias</i>	North Pacific	n/a			○		Verissimo et al. 2010

	North Atlantic	<i>n/a</i>			○	
	Southern Group	<i>n/a</i>			●	
<i>Stegastes partitus</i>	Bahamas	∞	LD			Christie et al. 2010
<i>Thunnus albacares</i>	Japan	8×10^3	BA	$5 \times 10^{-3} - 10^{-4}$	○	Qiu et al. 2010
<i>Thunnus orientalis</i>	Japan	6×10^3	BA		○	Riccioni et al. 2010
	Adriatic Sea	700	temp		○	
	Tyrrhenian Sea	400	temp		○	
<i>Urobatis halleri</i>	California, USA	<i>n/a</i>			●	Plank et al. 2010
<i>Urophycis tenuis</i>	Gulf of St. Lawrence	10^3 *	LD			Roy et al. 2012
	Newfoundland	$1-3 \times 10^3$ *	LD			
	Scotian Shelf	10^3-10^4 *	LD			
<i>Verasper variegatus</i>	Japan	<i>n/a</i>	temp		●	Sekino et al. 2011

* if upper CI ∞

BA: Bayesian approximation

Co: Coalescent

HE: heterozygote excess

LD: linkage disequilibrium

Ped: Pedigree

temp: temporal

References

- Ahonen H, Harcourt, RG, Stow, AJ (2009) Nuclear and mitochondrial DNA reveals isolation of imperilled grey nurse shark populations (*Carcharias taurus*). *Mol Ecol* **18**, 4409-4421.
- Allendorf FW (1986) Genetic drift and the loss of alleles versus heterozygosity. *Zoo Biol* **5**, 181-190.
- Amos W, Worthington WJ, Fullard K, Burg TM, Croxall JP, Block D, Coulson T (2001) The influence of parental relatedness on reproductive success. *P R Soc B* **268**, 2021-2027.
- Anderson JD, Karel WJ (2012) Population Genetics of Southern Flounder with Implications for Management. *North American Journal of Fisheries Management* **32**, 656-662.
- Aparicio JM, Ortego J, Cordero PJ (2006) What should we weigh to estimate heterozygosity, alleles or loci?. *Mol Ecol* **15**, 4659-4665.
- Araguas RM, Vidal O, Pla C, Sanz, N (2012) High genetic diversity of the endangered Iberian three-spined stickleback (*Gasterosteus aculeatus*) at the Mediterranean edge of its range. *Freshwater Biol* **57**, 143-154.
- Armbruster P, Reed DH (2005) Inbreeding depression in benign and stressful environments. *Heredity* **95**, 235-242.
- Avice JC (2000) Phylogeography: the history and formation of species. *Cambridge (MA): Harvard University Press*.
- Bagley MJ, Lindquist DG, Geller JB (1999) Microsatellite variation, effective population size, and population genetic structure of vermilion snapper, *Rhomboplites aurorubens*, off the southeastern USA. *Marine Biology* **134**, 609-620.
- Balazik MT, Garman GC, Fine ML, Hager CH, McIninch SP (2010) Changes in age composition and growth characteristics of Atlantic strugein (*Acipenser oxyrinchus oxyrinchus*) over 400 years. *Bio Letters* **6**, 708-710.
- Barraclough TGV (1998) Revealing the factors that promot speciation. *Phil Trans: Biol Sci* **353**, 241-249.
- Beaumont MA, Zhang W, Balding DJ (2002) Approximate Bayesian computation in population genetics. *Genetics* **162**, 2025-2035.
- Begon M (2006) Ecology: from individuals to ecosystems. *Blackwell Publishing 4th ed*, p. 5-8.
- Benton MJ (2001) Biodiversity on land and in the sea. *Geol. J.* **36**, 211-230.
- Berthier P, Beaumont MA, Cornuet J-M, Luikart G (2002) Likelihood-based estimation of the effective population size using temporal changes allele frequencies: A genealogical approach. *Genetics* **160**, 741-751.
- Betancur R (2010) Molecular phylogenetics supports multiple evolutionary transitions from marine to freshwater habitats in ariid catfishes. *Mol Phylogenet Evol* **55**, 249-258.
- Bickley LK, Brown AR, Hosken DJ, Hamilton PB, Le Page G, Paull GC, Owen SF, Tyler CR (2012) Interactive effects of inbreeding and endocrine disruption on reproduction in a model laboratory fish. *Evol Appl*
- Bierne N, Bonhomme F, David P (2003) Habitat preference and marine-speciation paradox. *Proc. R. Soc. Lond. Ser. B-Biol. Sci* **270**, 1399-1406.

- Bloom DD, Weir JT, Piller KR, Lovejoy NR (2013) Do freshwater fishes diversify faster than marine fishes? A test using state-dependent diversification analyses and molecular phylogenetics of new world silversides (Atherinopsidae). *Evolution*. DOI: 10.1111/evo.12074
- Bonfil R, Meyer M, Scholl MC, Johnson R, O'Brien S, Oosthuizen H, Swanson S, Kotze D, Paterson M (2005) Transoceanic migration, spatial dynamics, and population linkages of white sharks. *Science* **310**.
- Boreman J (1997) Sensitivity of North American sturgeons and paddlefish to fishing mortality. *Environ Biol Fishes* **48**, 399-405.
- Buckley J (1989) Species Profiles: Life histories and environmental requirements of coastal fishes and invertebrates (North Atlantic) - Winter Flounder. *U.S. Fish Wildlife Service Biological Report* Biological Report 82 (11.87), U.S. Army Corps of Engineers, TR EL-82-84 12 pp.
- Buckley J, Smigielski AS, Halavik TA, Caldarone EM, Burns BR, Laurence GC (1991) Winter flounder *Pseudopleuronectes americanus* reproductive success. I. Among-location variability in size and survival of larvae reared in the laboratory. *Marine Ecology Progress Series* **74**, 117-124.
- Buckley L, Collie J, Kaplan, LAE, Crivello J (2008) Winter Flounder Larval Genetic Population Structure in Narragansett Bay, RI: Recruitment to Juvenile Young-of-the-Year. *Estuar Coasts* **31**, 745-754.
- Busch JD, Waser PM, DeWoody JA (2007) Recent demographic bottlenecks are not accompanied by a genetic signature in banner-tailed kangaroo rats (*Dipodomys spectabilis*). *Mol Ecol* **16**, 2450-2462.
- Caballero A (1994) Developments in the prediction of effective population size. *Nature* **73**, 657-679.
- Capuis M-R, Estoup A (2007) Microsatellite null alleles and estimation of population differentiation. *Mol Biol Evol* **24**, 621-631.
- Carlsson J, Shephard S, Coughlan J, Trueman CN, Rogan E, Cross TF (2011) Fine-scale population structure in a deep-sea teleost (orange roughy, *Hoplostethus atlanticus*). *Deep Sea Research I* **58**, 627-636.
- Castric V, Bernatchez L, Belkhir K, Bonhomme F (2002) Heterozygote deficiencies in small lacustrine populations of brook charr *Salvelinus fontinalis* Mitchell (Pisces, Salmonidae): a test of alternative hypotheses. *Heredity* **89**, 27-35.
- North East Fisheries Center (2011) 52nd Northeast Regional Stock Assessment Workshop (52nd SAW) Assessment Report. *NEFSC Ref Doc 11-17;962 p*. Available from: National Marine Fisheries Service, 166 Water Street, Woods Hole, MA 02543-1026, or online at <http://nefsc.noaa.gov/publications>.
- Chant RJ, Curran MC, Able KW, Glenn SM (2000) Delivery of winter flounder (*Pseudopleuronectes americanus*) larvae to settlement habitats in coves near tidal inlets. *Estuar Coast Shelf Sci* **51**, 529-541.
- Chapman DD, Simpfendorfer C, Wiley TR, Poulakis GR, Curtis C, Tringali M, Carlson JK, Feldheim KA. (2011) Genetic diversity despite population collapse in a critically endangered marine fish: the smalltooth sawfish (*Pristis pectinata*). *J Her* **102**, 643-652.
- Chapman RW, Ball AO, Mash LR (2002) Spatial homogeneity and temporal heterogeneity of red drum (*Sciaenops ocellatus*) microsatellite: effective population sizes and management implications. *Mar Biotechnol* **4**, 589-603.

- Chapman TW, Stewart C (1996) Extremely high levels of inbreeding in a natural population of the free-living wasp *Ancistrocerus antilope* (Hymenoptera: Vespidae: Eumeniinae). *Heredity* **76**, 65-69.
- Chapple TK, Jorgensen SJ, Anderson SD, Kanive PE, Klimley AP, Botsford LW, Block BA (2011) A first estimate of white shark, *Carcharodon carcharias*, abundance off Central California. *Bio Letters* **7**, 581-583.
- Charlesworth B (2009) Effective population size and patterns of molecular evolution and variation. *Nat Rev Genet* **10**.
- Charlier J, Palme A, Laikre L, Andersson J, Ryman N (2011) Census (N_c) and genetically effectively (N_e) population size in a lake-resident population of brown trout *Salmo trutta*. *J Fish Biol* **79**, 2074-2082.
- Chevolot M, Ellis JR, Rijnsdorp AD, Stam WT, Olsen JL (2008) Temporal changes in allele frequencies but stable genetic diversity over the past 40 years in the Irish Sea population of thornback ray, *Raja clavata*. *Heredity* **101**, 120-126.
- Christie MR, Johnson DW, Stallings CD, Hixon MA (2010) Self-recruitment and sweepstakes reproduction amid extensive gene flow in a coral-reef fish. *Mol Ecol* **19**, 1042-1057.
- Cliff G, van der Elst R, Govender A, Witthuhn TK, Bullen, EM (1996) First estimates of mortality and population size of white sharks on the South African coast. In *Great white sharks: the biology of Carcharodon carcharias*, 393-400.
- Compagno LJV, Marks MA, Fergusson IK (1997) Threatened fishes of the world: *Carcharodon carcharias* (Linnaeus, 1758) (Lamnidae). *Environ Biol Fish* **50**.
- Conover DM, Munch S, Lankford Jr T (2000a) Current status of the Key silverside, *Menidia menidia*, in southern Florida. Final Project Report. U.S. Geological Survey Biological Resources Division Restoration Ecology Branch, Florida Caribbean Science Center, Florida International University, Miami, FL 33199. 18p.
- Conover DO (1998) Local adaptation in marine fishes: Evidence and implications for stock enhancement. *B Mar Sci* **62**, 477-493.
- Cornuet JM, Luikart G (1996) Description and power of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics* **144**, 2001-2014.
- Cornuet JM, Santos F, Beaumont MA, Robert CP, Marin J-M., Balding DJ, Guillelmo T, Estoup, A (2008) Inferring population history with DIY ABC: a user-friendly approach to approximate Bayesian computation. *Bioinformatics* **24**, 2713-2719.
- Coscia I, Vogiatzi E, Kotoulas G, Tsigenopoulos CS, Mariani S (2011) Exploring neutral and adaptive processes in expanding populations of gilthead sea bream, *Sparus aurata* L., in the North-East Atlantic. *Heredity* **108**, 537-546.
- Cowen RK, Sponaugle S (2009) Larval dispersal and marine population connectivity. *Annual Review Marine Science* **1**, 443-446.
- Coyne JA (1992) Genetics and speciation. *Nature* **355**.
- Cracraft J (1982) A nonequilibrium theory for the rate-control of speciation and extinction and the origin of macroevolutionary patterns. *Syst Zool* **31**, 348-365.
- Crivello JF, Danila DJ, Lorda E, Keser M, Roseman EF (2004) The genetic stock structure of larval and juvenile winter flounder larvae in Connecticut waters of eastern Long Island Sound and estimations of larval entrainment. *J Fish Biol* **65**, 62-76.

- Cronin M, Armstrup SC, Talbot SL, Sage GK, Armstrup S (2009) Genetic variation, relatedness, and effective population size of polar bears (*Ursus maritimus*) in the southern Beaufort Sea, Alaska. *J Her* **100**, 681-690.
- Cuveliers EL, Volkaert FAM, Rijnsdorp, AD, Larmuseau HD, Maes GE. (2011) Temporal genetic stability and high effective population size despite fisheries-induced life-history trait evolution in the North Sea sole. *Mol Ecol* **20**, 3555-3568.
- DeHaan PW, Libants SV, Elliot RF, ScribnerKT (2006) Genetic population structure of remnant Lake sturgeon populations in the Upper Great Lakes Basin. *TAFS* **135**, 1478-1492.
- DeLong AK, Collie JS, Meise CJ, Powell JC (2001) Estimating growth and mortality of juvenile winter flounder, *Pseudopleuronectes americanus*, with a length-based model. *Can J Fish Aquat Sci* **58**, 2233-2246.
- DeWoody JA, Avise JC. (2000) Microsatellite variation in marine, freshwater and anadromous fishes compared with other animals. *J Fish Biol***56**, 461-473.
- Dhamarajan G, Beatty WS, Rhodes Jr OE (2012) Heterozygote deficiencies caused by a Wahlund effect: Dispelling unfounded expectations. *J Wildlife Manage* **77**, 226-234.
- Dinerstein E, McCracken GF (1990) Endangered greater one-horned rhinoceros carry high levels of genetic variation. *Conserv Biol* **4**, 417-422.
- Dongdong X, Sanlei LI, Bao L, Yurong Z, Wei Z, Huilai S (2012) Genetic diversity in two Japanese flounder populations from China seas inferred using microsatellite markers and COI sequences. *Chinese Journal of Oceanology and Limnology* **30**, 604-610.
- Duggins Jr. C, Karlin A, Relyea K, Yerger, R (1986) Systematics of the Key silverside *Menidia conchorum* with comments on other *Menidia* species (Pisces Atherinidae). *Tulane Studies in Zoology and Botany* **25**, 133-150.
- Dulvy NK, Baum JK, Clarke S, Compagno LJV, Cortés E, Domingo A, Fordham S, Fowler S, Francis MP, Gibson C, Martínez J, Musick JA, Soldo A, Stevens JD, Valenti S (2008) You can swim but you can't hide: the global status and conservation of oceanic pelagic sharks and rays. *Aquatic Conserv: Mar. Freshw. Ecosyst.* **18**, 459-482.
- Dunton KJ, Chapman DD, Jordaan A, Feldheim K, O'Leary SJ, McKnown KA, Frisk MG (2011) Genetic mixed-stock analysis of Atlantic sturgeon *Acipenser oxyrinchus oxyrinchus* in a heavily exploited marine habitat indicates the need for routine genetic monitoring. *J Fish Biol* **80**, 207-217.
- Dunton KJ, Jordaan A, McKown KA, Conover DO, Frisk MG (2010) Abundance and distribution of Atlantic sturgeon (*Acipenser oxyrinchus*) within the North Atlantic Ocean, determined from five fishery-independent surveys. *Fish B-NOAA* **108**.
- Duong TY, Scribner KT, Forsythe PS, Crossman JA, Baker EA (2013) Interannual variation in effective number of breeders and estimation of effective population size in long-lived iteroparous lake sturgeon (*Acipenser fulvescens*). *Mol Ecol* **22**, 1282-1294.
- Engen S, Lande R, Saether BE (2005) Effective size of a fluctuating age-structured population. *Genetics* **170**, 941-954.
- England PR, Osler GHR, Woodworth LM, Montgomery ME, Briscoe DA, Frankham R (2003) Effects of intense versus diffuse population bottlenecks on microsatellite genetic diversity and evolutionary potential. *Conserv Gen* **4**, 595-604.

- Eschmeyer WN, Fricke R, Fong JD, Polack DA (2010) Marine fish diversity: history of knowledge and discovery (Pisces). *Zootaxa*, 19-20.
- Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software Structure: a simulation study. *Mol Ecol* **14**, 2611-2620.
- Excoffier L, Lisher HEL (2010) Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Mol Ecol R* **10**, 564-567.
- Ferguson PL, Iden CR, McElroy AE, Brownawell BJ (2001) Determination of steroid estrogens in wastewater by immunoaffinity extraction couples with HPLC-MS. *Anal. Chem* **731**, 3890-3895.
- Florin A-B, Hoglund J (2007) Absence of population structure of turbot (*Psetta maxima*) in the Baltic Sea. *Mol Ecol* **16**, 115-126.
- Florin A-B, Hoglund J (2008) Population structure of flounder (*Platichthys flesus*) in the Baltic Sea: differences among demersal and pelagic spawners. *Heredity* **101**, 27-38.
- Frankham R (1996) Relationship of Genetic Variation to Population Size in Wildlife. *Conserv Biol* **10**, 1500-1508.
- Frankham R (1997) Do island populations have less genetic variation than mainland populations? *Heredity* **78**, 311-327.
- Frankham R (1998) Inbreeding and Extinction: Island populations. *Conserv Biol* **12**, 665-675.
- Frankham R, Lees K, Montgomery ME, England PR, Lowe EH, Briscoe DA (1999) Do population size bottlenecks reduce evolutionary potential?. *Anim Conserv* **2**, 255-260.
- Franklin IR, Frankham R (1998) How large must populations be to retain evolutionary potential? *Anim Conserv* **1**, 69-73.
- Frasier TR (2008) STORM: software for testing hypotheses of relatedness and mating patterns. *Mol Ecol Resources* **8**, 1263-1266.
- Frisk MG, Jordaan A, Miller TJ (2013) Moving beyond the current paradigm in marine population connectivity: are adults the missing link?. *Fish Fish*.
- Garza JC, Williamson E (2001) Detection of reduction in population size using data from microsatellite loci. *Mol Ecol* **10**, 305-318.
- Getter C (1981) Ecology and survival of the Key silverside, *Menidia conchorum*, an atherinid fish endemic to the Florida Keys. *PhD Dissertation. University of Miami*.
- Gilpin M (1991) The genetic effective size of a metapopulation. *Biological Journal of the Linnean Society* **42**, 165-175.
- Glenn TC, Schnable NA (2005) Isolating microsatellite DNA loci. *Methods Enzymol* **395**, 202-222.
- Gomez-Uchida D, Banks MA (2006) Estimation of effective population size for the long-lived darkblotched rockfish *Sebastes cramerii*. *J Her* **97**, 603-606.
- Goudet J (1995) Fstat version 1.2: a computer program to calculate Fstatistics. *J Her* **86**, 485-486.
- Hauser L, Adcock GJ, Smith J, Bernal-Ramirez JH, Carvalho GR (2002) Loss of microsatellite diversity and low effective population size in an overexploited population of New Zealand snapper (*Pagrus auratus*). *PNAS* **99**, 11742-11747.

- Grogan CS, Boreman J (1998) Estimating the probability that historical populations of fish species are extirpated. *North American Journal of Fisheries Management* **18**, 522-529.
- Grunwald C, Maceda L, Waldman J, Stabile J, Wirgin I (2008) Conservation of Atlantic sturgeon *Acipenser oxyrinchus oxyrinchus*: delineation of stock structure and distinct population segments. *Conserv Genet* **9**, 1111-1124.
- Gubili C, Bilgin R, Kalkan E, Karhan SU. (2010) Antipodean white sharks on a Mediterranean walkabout? Historical dispersal leads to genetic discontinuity and an endangered anomalous population. *Proc Roy Soc B* **278**, 1679-1686.
- Guinand B, Frustier MA, Labonne M, Jourdain E, Calves I, Quiniou L, Cerqueira F, Laroche J (2013) Genetic structure and heterozygosity-fitness correlation in young-of-the-year sole (*Solea solea* L.) inhabiting three contaminated West-European estuaries. *J Sea Research* **80**, 35-49.
- Haffray P, Malha R, Ould Taleb Sidi M, Prista N, Hassan M, Castelnaud G, Karahan-Nomm B, Gamiz K, Sadek S, Bruant J-S, Balma P, Bonhomme F (2012) Very high genetic fragmentation in a large marine fish, the meagre *Argyrosomus regius* (Sciaenidae, Perciformes) impact of reproductive migration, oceanographic barriers and ecological factors. *Aquatic Living Resources* **25**, 173-183.
- Hailer F, Helande B, Folkestad AO, Ganusevich SA, Garstad S, Hauff P, Koren C, Nygard T, Volke V, Vila C, Ellegren H (2006) Bottlenecked but long-lived: high genetic diversity retained in white-tailed eagles upon recovery from population decline. *Bio Letters* **2**, 316-319.
- Hale ML, Burg TM, Steeves TE (2012) Sampling for microsatellite-based population genetic studies: 25 to 30 individuals per population is enough to accurately estimate allele frequencies. *PLOS One* **7**, e45170.
- Hare MP, Nunney L, Schwartz MK, Ruzzante DE, Burford M, Waples RS, Ruegg K, Palstra F (2011) Understanding and estimating effective population size for practical application in marine species management. *Conserv Biol* **25**, 438-449.
- Hauser L, Carvalho GR (2008) Paradigm shifts in marine fisheries genetics: ugly hypotheses slain by beautiful facts. *Fish Fish* **9**, 333-362.
- Hemmer-Hansen J, Nielsen EEG, Gronkjaer P, Loeschke V (2007) Evolutionary mechanisms shaping the genetic population structure of marine fisheries; lessons from the European flounder (*Platichthys flesus* L.). *Mol Ecol* **16**, 3104-3118.
- Henderson-Arzapalo A, King TL (2002) Novel microsatellite markers for Atlantic sturgeon (*Acipenser oxyrinchus*) population delineation and broodstock management. *Mol Ecol* **2**, 437-439.
- Hendrickson L, Nitschke P, Terceiro M (2006) Winter flounder. *NOAA Northeastern Fisheries Science Center Stock Assessment*, 30 pp.
- Hildebrand S, Ginsburg I (1927) Descriptions of two new species of fishes from Key West, Florida with notes on nine other fishes collected in the same locality. *Fisheries Bulletin US Fish and Wildlife Service* **42**, 207-215.
- Hoarau G, Boon E, Jongma DN, Ferber S, Palsson J, Van der Veer HK, Rijnsdorp AD, Stam WT, Olsen JL (2005) Low effective population size and evidence for inbreeding in an overexploited flatfish, plaice (*Pleuronectes platessa* L.). *P R Soc B* **272**, 497-503.
- Hoehn M, Gruber B, Sarre SD, Lange R, Henle K (2012) Can genetic estimators provide robust estimates of effective number of breeders in small populations. *PLOS One* **7**, e48464.

- Hoelzel AR, Shivji M, Magnussen J, Francis MP (2006) Low worldwide genetic diversity in the basking shark (*Cetorhinus maximus*). *Bio Letters* **2**, 639-642.
- Hoffmeister JE, Multer HG (1968) Geology and Origin of the Florida Keys. *Geol Soc Am Bull* **79**, 1487-1502.
- Horn MH (1972) The amount of space available for marine and freshwater fishes. *Fish Bull U S* **70**, 1295-1297.
- Hughes JM, Real KM, Marshall JC, Schmidt DJ (2012) Extreme genetic structure in small-bodied freshwater fish, the purple spotted gudgeon, *Mogurnda adspersa* (Eleotridae). *PLOS One* **7**, e40546.
- Hutchings JA, Minto C, Ricard D, Baum JK, Jensen OP (2010) Trends in the abundance of marine fishes. *Can J Fish Aquat Sci* **67**, 1205-1210.
- Hutchings JA, Reynolds JD (2004) Marine fish population collapses: Consequences for recovery and extinction risk. *BioScience* **54**, 297-309.
- Hutchinson WF, van Oosterhout C, Rogers SI, Carvalho GR (2003) Temporal analysis of archived samples indicates marked genetic changes in declining North Sea cod (*Gadus morhua*). *Proc Roy Soc B* **270**, 2125-2132.
- Huxley T (1883) Inaugural Address. Fisheries Exhibition, London.
- Jacquard A (1975) Inbreeding: one word, several meanings. *Theoretical Population Biology* **7**, 338-363.
- Jarne P, Lagoda PJJ (1996) Microsatellites, from molecules to populations and back. *Ecol Evol* **11**, 424-429.
- Jensen JL, Bohonak AJ, Kelley ST (2005) Isolation by distance, web service. *BMC Genetics* **6**, 13.
- Johnson JA, Tingay R, Culver M, Hailer F, Clarke M, Mindell DP. (2009) Long-term survival despite low genetic diversity in the critically endangered Madagascar fish-eagle. *Mol Ecol* **18**, 54-63.
- Jorde PE, Ryman N (1995) Temporal allele frequency change and estimation of effective size in populations with overlapping generations. *Genetics* **139**.
- Jorde PE, Ryman N (2007) Unbiased estimator for genetic drift and effective population size. *Genetics* **177**, 927-935.
- Jorgensen SJ, Reeb CA, Chapple TK, Anderson S, Perle C, van Sommeran SR, Fritz-Cope C, Brown AC, Klimley AP, Block BA (2010) Philopatry and migration of Pacific white sharks. *Proc Roy Soc B* **277**, 679-688.
- Kahnle A W, Hatalla KA, McKown KA (2007) Status of Atlantic sturgeon of the Hudson River Estuary, New York, USA. *American Fisheries Society Symposium* **56**, 347.
- Kalinowski ST, Manlove KR, Taper ML. (2008) Oncor: a computer program for genetic stock identification, v. 2. *Department of Ecology, Montana State University, Bozeman, USA; 2008*.
- Karl SA, Castro ALF, Lopez JA, Charvet P, Burgess GH (2011) Phylogeography and conservation of the bull shark (*Carcharhinus leucas*) inferred from mitochondrial and microsatellite DNA. *Conserv Gen* **12**, 371-382.
- Karlsson S, Mork J (2005) Deviations from Hardy-Weinberg equilibrium and temporal instability in allele frequencies at microsatellite loci in a local population of Atlantic cod. *ICES Journal of Marine Science* **62**, 1588-1596.

- Keller IF, Waller DM (2002) Inbreeding effects in wild populations. *Trends in Ecol Evol* **17**, 230-241.
- Kenchington E, Heino M (2002) Maintenance of genetic diversity: challenges for management of marine resources. *ICES Journal of Marine Science* **Y:13**, 1-14.
- King TL, Lubinski BA, Spidle AP (2001) Microsatellite DNA variation in Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus*) and cross-species amplification in the Acipenseridae. *Conserv Gen* **2**, 103-119.
- Kocik J, Lipski C, Miller T, Rago P, Shepherd G (2013) An Atlantic Sturgeon Population Index for ESA Management Analysis. *US Dept Commer, Northeast Fish Sci Cent Ref Doc. 13-06*; 36p.
- Kubota H, Watanabe K, Suguro N, Tabe M, Umezawa K, Watanabe S (2010) Genetic population structure and management units of the endangered Tokyo bitterling, *Tanakia tanago* (Cyprinidae). *Conserv Gen* **11**, 2343-2355.
- Kuo C-H, Janzen FJ (2003) Bottlesim: a bottleneck simulation program for long-lived species with overlapping generations. *Mol Ecol Notes* **3**, 669-673.
- Kuo C-H, Janzen FJ (2004) Genetic effects of a persistent bottleneck on a natural population of ornate box turtles (*Terrapene ornata*). *Conserv Gen* **5**, 425-437.
- Lacy RC (1987) Loss of genetic diversity from managed populations: interacting effects of drift, mutation, immigration, selection, and population subdivision. *Conserv Biol* **1**, 143-158.
- Lande R (1980) Genetic variation and phenotypic evolution during allopatric speciation. *The American Naturalist* **116**, 463-479.
- Langen K, Schwarzer J, Kullmann H, Bakker TCM, Thunken T (2011) Microsatellite support for active inbreeding in a cichlid fish. *PLOS One* **6**, 1-9.
- Larsson LC, Laikre L, Andre C, Dahlgren TG, Ryman N (2010) Temporally stable genetic structure of heavily exploited Atlantic herring (*Clupea harengus*) in Swedish waters. *Heredity* **104**, 40-51.
- Latch EK, Dharmajan G, Glaubitz JC, Rhodes OE (2006) Relative performance of Bayesian clustering software for inferring population substructure and individual assignment at low levels of population differentiation. *Conserv Gen* **7**, 295-302.
- Laurent V, Planes S (2007) Effective population size estimation on *Sardina pilchardus* in the Bay of Biscay using a temporal genetic approach. *Biological Journal of the Linnean Society* **90**, 591-602.
- Leberg PL (2005) Genetic approaches for estimating the effective size of populations. *J Wildlife Manage* **69**, 1385-1399.
- Lemer S, Rochel E, Planes S (2011) Correction method for null alleles in species with variable microsatellite flanking regions, a case study of the black-lipped pearl oyster *Pinctada margaritifera*. *J Her* **2011**, 2.
- Lerner HRL, Johnson JA, Lindsay AR, Kiff LF, Mindell DP (2009) It's not too late for the harpy eagle (*Harpia haryja*): High levels of genetic diversity and differentiation can fuel conservation programs. *PLoS ONE* **4**, e7336.
- Leveque C, Oberdorff T, Paugy D, Stiassny MLJ, Tedesco PA (2008) Global diversity of fish (Pisces) in freshwater. *Hydrobiologia* **595**, 545-567.

- Liao W, Reed DH (2009) Inbreeding-environment interactions increase extinction risk. *Anim Conserv* **12**, 54-61.
- Liberg O, Andren H, Pedersen H-C, Sand H, Sejberg D, Wabakken P, Akesson M, Bensch S (2005) Severe inbreeding depression in a wild wold *Canis lupus* population. *Bio Letters* **1**, 17-20.
- Limborg MT, Hanel R, Debes PV, Ring AK, Andre C, Tsigenopoulos CS, Bekkevold D (2012) Imprints from genetic drift and mutation imply relative divergence times across marine transition zones in a pan-European small pelagic fish (*Sprattus sprattus*). *Heredity* **109**, 96-107.
- Lippe C, Dumont P, Bernatchez L (2006a) High genetic diversity and no inbreeding in the endangere copper redhorse, *Moxostoma hubbsi* (Catostomidae, Pisces): the positive sides of a long generation time. *Mol Ecol* **15**, 1769-1780.
- Lobell MJ (1939) A biological survey of the salt waters of Long Island, 1938. Report on certain fishes. Winter Flounder (*Pseudopleuronectes americanus*). *28th Ann Rep NY St Cons Dept. Suppl., Pt. 1, pp 63-90.*
- Lotterhos KE, Markel RW (2012) Oceanographic drivers of offspring abundance may increase or decrease reproductive variance in a temperate marine fish. *Mol Ecol* **21**, 5009-5026.
- Luikart G, Allendorf F, Cornuet JM, Sherwin W (1998) Distortion of allele frequency distributions provides a test for recent population bottlenecks. *J Her* **89**, 238-247.
- Luikart G, Ryman N, Tallmoon DA, Schwartz MK, Allendorf F (2010) Estimation of census and effective population sizes: the increasing usefulness of DNA-based approaches. *Conserv Gen* **11**, 355-373.
- Lundberg JG, Kottelat M, Smith GR, Stiassny MLJ, Gill AC (2000) So many fishes, so little time: an overview of recent ichthyological discovery in continental waters. *Ann. Missouri Botanical Garden* **87**, 26-62.
- Lynch M, Lande R (1998) The critical effective size for a genetically secure population. *Anim Conserv* **1**, 70-72.
- Macbeth GM, Broderick D, Buckworth RC, Ovenden JR (2013) Linkage disequilibrium estimation of effective population size with immigrants from divergent populations: a case study on Spanish mackerel (*Scomberomorus commerson*). *G3*.
- Mantel N (1967) The detection of disease clustering and generalized regression approach. *Cancer Re* **27**.
- Mariani S, Hutchinson WF, Hatfield EMC Ruzzante DE Simmonds EJ, Dahlgren TG, Andre C, Brigham J, Torstensen E, Carvalho GR (2005) North Sea herring population structure revealed by microsatellite analysis. *Marine Mol Ecol Progress Series* **303**, 245-257.
- May RM (1994a) Biological diversity: differences between land and sea. *Phil Trans R Soc Lond Ser B Biol Sci* **343**, 105-111.
- McCairns RJS, Kuparinen A, Panda B, Jokikokko E, Merila J (2012) Effective size and genetic composition of two exploited, migratory whitefish (*Coregonus lavaretus lavaretus*) populations. *Conserv Gen*.
- McClelland G, Melendy J, Osborne H, Reid D, Douglas S (2005) Use of parasite and genetic markers in delineating populations of winter flounder from the central and south-west Scotian Shelf and north-east Gulf of Maine. *J Fish Biol* **66**, 1082-1100.
- McCracken FD (1963) Seasonal movements of the winter flounder, *Pseudopleuronectes americanus* (Walbaum), on the Atlantic coast. *TAFS* **34**, 749-752.

- McNeely JA, Miller KRW, Reid WV, Mittermeier RA, Werner TB (1990) Conserving the world's biological diversity. International Union for Conservation of Nature and Natural Resources, World Resources Institute, Conservation International, World Wildlife Fund-US, and the World Bank, Gland, Switzerland, and Washington, D.C. .
- Mena L, Arukwe A, Desnlow D, McElroy A (2006) Endocrine disruption in winter flounder (*Pseudopleuronectes americanus*) from an urban estuary, Jamaica Bay, NY USA. *Mar Environ Res* **62**, S223-S234.
- Miotto RA, Cervini M, Figueiredo MG, Begotti RA, Galetti PM. (2011) Genetic diversity and population structure of pumas (*Puma concolor*) in southeastern Brazil: implications for conservation in a human-dominated landscape. *Conserv Gen* **12**, 1447-1455.
- Mourier J, Planes S (2013) Direct genetic evidence for reproductive philopatry and associated fine-scale migrations in female blacktip reef sharks (*Carcharhinus melanopterus*) in French Polynesia. *Mol Ecol* **22**, 201-214.
- Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* **89**, 583-590.
- Nei M, Maruyama T, Chakraborty R (1975) The bottleneck effect and genetic variability in populations. *Evolution* **29**, 1-10.
- Nei M, Tajima F (1981) Genetic drift and estimation of effective population size. *Genetics* **98**, 3.
- Nielsen JL, Graziano SL, Seitz AC (2010) Fine-scale population genetic structure in Alaskan Pacific halibut (*Hippoglossus stenolepis*). *Conserv Gen* **11**, 999-1012.
- NMFS (2010) Endangered and threatened wildlife; notice of 90-day finding on a petition to list Atlantic Sturgeon as threatened or endangered under the Endangered Species Act (ESA). *Federal Register* **75**, 838-841.
- Norouzi M, Nazemi A, Pourkazemi M (2012) Population genetic study in common kilka (*Clupeonella cultriventris* Nordman, 1840) in the Southwest Caspian Sea (Gilan Province, Iran) using microsatellite markers. *African Journal of Biotechnology* **11**, 16405-16411.
- Nunney L (1993) The influence of mating system and overlapping generations on effective population size. *Evolution* **47**, 1329-1341.
- O'Grady JJ, Book BW, Reed DH, Ballou JD, Tonkyn DW, Frankham R (2006) Realistic levels of inbreeding depression strongly affect extinction risk in wild populations. *Biol Conserv* **133**, 42-51.
- O'Leary SJ, Feldheim KA, Chapman DD (2013) Novel microsatellite loci for white, *Carcharodon carcharias* and sandtiger sharks, *Carcharias taurus* (order Lamniformes). *Conserv Genet Resour*
- O'Leary SJ, Feldheim KA, Chapman DD (2013a) Novel microsatellite loci for winter flounder (*Pseudopleuronectes americanus*). *Conserv Gen Resour*
- O'Leary SJ, Hice LA, Feldheim KA, Frisk MG, McElroy AE, Fast MD, Chapman DD (2013b) Severe inbreeding and small effective number of breeders in a formerly abundant marine fish. *PLOS One* **8**, e66126.
- Ong T-L, Stabile J, Wirgin I, Waldman JR (1996) Genetic divergence between *Acipenser oxyrinchus oxyrinchus* and *A. o. desotoi* as assessed by mitochondrial sequencing analysis. *Copeia* **1996**, 464-469.

- Van Oosterhout C, Hutchinson WF, Wills DPN, Shipley P (2004) Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. *Mol Ecol Notes* **4**, 535-538.
- Palof KJ, Heifetz J, Gharrett AJ (2011) Geographic structure in Alaskan Pacific ocean perch (*Sebastes alutus*) indicates limited lifetime dispersal. *Marine Biology* **158**, 779-792.
- Palstra FP, Fraser DJ (2012) Effective/Census population size ratio estimation: a compendium and appraisal. *Ecol Evol*.
- Palstra FP, Ruzzante DE (2008) Genetic estimation of contemporary effective population size: what can they tell us about the importance of genetic stochasticity for wild population persistence? *Mol Ecol* **17**, 3428-3447.
- Palumbi SR (1994) Genetic divergence, reproductive isolation, and marine speciation. *Annu. Rev. Ecol. Syst.* **25**, 547-572.
- Pardini AT, Jones CS, Noble LR, Kreiser B, Malcolm H, Bruce BD, Stevens JD, Cliff G, Scholl MC, Francis M, Duffy C, Martin AP (2001) Sex-biased dispersal of great white sharks. *Nature* **412**, 139-140.
- Park SDE (2001) Trypanotolerance in West African Cattle and the Population Genetic Effects of Selection. *PhD Thesis* University of Dublin.
- Peel D, Ovenden JR, Peel SL (2004) NeEstimator: software for estimating effective population size, Version 1.3. *Queensland Government, Department of Primary Industries and Fisheries*.
- Peery MZ, Kirby R, Reid BN, Stoelting R, Doucet-Beer E, Robinson S, Vasquez-Carillo C, Pauli JN, Palsboll PJ (2012) Reliability of genetic bottleneck tests for detecting recent population declines. *Mol Ecol* **21**, 3403-3418.
- Perlmutter A (1947) The blackback flounder and its fishery in New England and New York. *Bulleting of the Bingham Oceanographic Collection* **11**, 1-92.
- Peterson DL, Schueller P, DeVries Rm Fleming J, Grunwald C, Wirgin I (2008) Annual run size and genetic characteristics of Atlantic sturgeon in Altamaha River, Georgia. *TAFS* **137**, 393-401.
- Pikitch EK, Doukakis P, Chakrabarty P, Erickson DL (2005) Status, trends and management of sturgeon and paddlefish fisheries *Fish Fish* **6**, 233-265.
- Piry S, Luikart G, Cornuet JM (1999) Bottleneck: a computer program for detecting recent reductions in the effective population size using allele frequency data. *J Her* **90**, 502-503.
- Plank SM, Lowe CG, Feldheim KA, Wilson Jr RR, Brusslan JA (2010) Population genetic structure of the round stingray *Urobatis halleri* (Elasmobranchii: Rajiformes) in southern California and the Gulf of California. *J Fish Biol* **77**, 329-340.
- Poole JC (1966) Growth and age of winter flounder in four bays of Long Island. *New York Fish Game J* **13**, 206-220.
- Portnoy DSH, C. M.; Renshaw, M. A.; Cummings, N. J.; Gold, J. R. (2013) Does mating behaviour affect connectivity in marine fishes? Comparative population genetics of two protogynous groupers (Family Serranidae). *Mol Ecol* **22**, 301-313.
- Portnoy DS, McDowell JR, McCandless CT, Musick JA, Graves JE (2009) Effective size closely approximates the census size in the heavily exploited western Atlantic population of the sandbar shark, *Carcharias plumbeus*. *Conserv Gen* **10**, 1697-1705.
- Poulsen NA, Nielsen EE, Schierup MH, Loeschcke V, Gronkjaer P (2006) Long-term stability and effective population size in North Sea and Baltic Sea cod (*Gadus morhua*). *Mol Ecol* **15**, 321-331.

- Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics* **155**, 945-959.
- Pudovkin AI, Zaykin DV, Hedgecock D (1996) On the potential for estimating the effective number of breeders from heterozygote-excess in progeny. *Genetics* **144**, 383-387.
- Puebla O (2009) Ecological speciation in marine v. freshwater fishes. *J Fish Biol* **75**, 960-996.
- Pujolar JM, Bevacqua D, Capoccioni F, Ciccotti E, De Leo GA, Zane L (2011) No apparent genetic bottleneck in the demographically declining European eel using molecular genetics and forward-time simulations. *Conserv Gen* **12**, 813-825.
- Qiu F, Miyamoto MM (2011) Use of nuclear DNA data to estimate genetic diversity and population size in Pacific Bluefin and Yellowfin Tuna (*Thunnus orientalis* and *T. albacares*). *Copeia* **2**, 264-269.
- Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Heredity* **86**, 2480249.
- Raymond M, Vaanto RL, Thomas F, Rousset F, de Meeus T, Renaud F (1997) Heterozygote deficiency in the mussel *Mytilus edulis* species complex revisited. *Marine Ecol Progr Series* **156**.
- Reed DH (2004) Extinction risk in fragmented habitats. *Anim Conserv* **7**, 181-191.
- Reynolds JD, Dulvy NK, Goodwin NB, Hutchings JA (2005) Biology of extinction risk in marine fishes. *Proc R Soc B* **272**, 2337-2344.
- Riccioni G, Landi M, Ferrara G, Milano I, Cariani A, Zane L, Sella M, Barbujani G, Tinti F (2010) Spatio-temporal population structuring and genetic diversity retention in depleted Atlantic bluefin tuna of the Mediterranean Sea. *PNAS* **107**, 2102-2107.
- Robinson JD, Simmons JW, Williams AS, Moyer GR (2013) Population structure and genetic diversity in the endangered bluemask darter (*Etheostoma akatulo*). *Conserv Gen* **14**, 79-92.
- Rousset F (2008) Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. *Mol Ecol Resources* **8**, 103-106.
- Roy D, Hurlbut TR, Ruzzante DE (2012) Biocomplexity in a demersal exploited fish, white hake (*Urophycis tenuis*): depth-related structure and inadequacy of current management approaches. *Can J Fish Aquat Sci* **69**, 415-429.
- Ruggeri P, Spelndiani A, Bonanomi S, Arneri E, Cingolani N, Santojanni A, Belardinelli A, Giovannotti M, Caputo V (2012) Temporal genetic variation as revealed by a microsatellite analysis of European sardine (*Sardina pilchardus*) archived samples. *Can J Fish Aquat Sci* **69**, 1698-1709.
- Sagarese SR, Frisk MG (2011) Movement patterns and residence of adult winter flounder within a Long Island estuary. *Marine and Coastal Fisheries: Dynamics, Management, and Ecosystem Science* **3**, 295-306.
- Saillant E, Gold JR (2006) Population structure and variance effective size of red snapper (*Lutjanus campechanus*) the northern Gulf of Mexico. *Fish B-NOAA* **104**, 136-148.
- Sanz N, Fernandez-Cebrian R, Casals F, Araguas RM, Garcia-Marin JL (2011) Dispersal and demography of brown trout, *Salmo trutta*, inferred from population and family structure in unstable Mediterranean streams. *Hydrobiologica* **671**, 105-119.
- Sbrocco EJ, Barber PH (2011) Ten polymorphic microsatellite loci for the Atlantic Silverside, *Menidia menidia*. *Conserv Gen Resources* **3**, 585-587.

- Schueller P, Peterzon DL (2010) Abundance and recruitment of juvenile Atlantic sturgeon in the Altamaha River, Georgia. *TAFS* **139**, 1526-1535.
- Secor DH (2002) Atlantic sturgeon fisheries and stock abundances during the late nineteenth century. *American Fisheries Society Symposium* **28**: 89-98.
- Secor DH, Waldman JR (1999) Historical abundance of Delaware Bay Atlantic sturgeon and potential rate of recovery. *American Fisheries Society Symposium* **23**, 203-216.
- Segvic-Bubic T, Lepen I, Trimbic, Y, Ljubkovic J, Sutlovic D, Matic-Skoko S, Grubisic L, Glamuzina B, Mladineo I (2011) Population genetic structure of reared and wild gilthead sea bream (*Sparus aurata*) in the Adriatic Sea inferred with microsatellite loci. *Aquaculture* **318**, 309-315.
- Sekino M, Saitoh K, Shimizu D, Wada T, Kamiyama K, Gambe S, Chen S, Aritaki M (2011) Genetic structure in species with shallow evolutionary lineages: a case study of the rare flatfish *Verasper variegatus*. *Conserv Gen* **12**, 139-159.
- NNMF (2007) Annual commercial landing statistics, NOAA Fisheries, Office of Science and Technology, Fisheries Statistics and Economics, Washington, DC. URL: http://www.st.nmfs.noaa.gov/st1/commercial/landings/annual_landings.html.
- Seymour M, Rasanen K, Holderegger R, Kristjansson BK (2013) Connectivity in a pond system influences migration and genetic structure in threespine stickleback. *Ecol Evol* **3**, 492-502.
- Shrey AW, Heist EJ (2002) Microsatellite markers for the shortfin mako and cross-species amplification in lamniformes. *Conserv Gen* **3**, 459-461.
- Shunter C, Carreras-Carbonell J, Planes S, Sala E, Ballesteros E, Zabala M, Harmelin J-G, Harmelin-Vivien M, Macpherson E, Pascual M (2011) Genetic connectivity patterns in an endangered species: The dusky grouper (*Epinephelus marginatus*). *J Exp Mar Biol Ecol* **401**, 126-133.
- Slate JD, P.; Dodds, K. G.; Veenvliet, B. A.; Glass, B. C.; Broad, T. E.; McEwan, J. C. (2004) Understanding the relationship between the inbreeding coefficient and multilocus heterozygosity: theoretical expectations and empirical data. *Heredity* **93**, 255-265.
- Slate J, Kruuk LEB, Marshall TC, Pemberton JM, Clutton-Brock TH (2000) Inbreeding depression influences lifetime breeding success in a wild population of red deer (*Cervus elaphus*). *P R Soc B* **267**, 1657-1662.
- Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. *Genetics* **139**, 457-462.
- Smedbol RK, McPherson A, Hansen MM, Kenchington E (2002) Myths and moderation in marine 'metapopulations'?. *Fish Fish* **44**, 213-232.
- Smith TIJ, Clugston JP (1997) Status and management of Atlantic sturgeon, *Acipenser oxyrinchus*, in North America. *Environ Biol Fish* **48**, 335-346.
- Socrates JB (2006) A study of the striped bass in the marine district of New York State. New York State Department of Environmental Conservation, East Setauket, NY Completion Report for Project AFC-30.
- Sosa-Nishizaki O, Morales-Bojorquez E, Nasby-Lucas N, Onata-Gonzalez EC, Domeier ML (2012) Problems with photo identification as a method of estimating abundance of white sharks, *Carcharodon carcharias*. *Global Perspectives on the Biology and Life History of the Great White Shark*, CRC Press, Boca Raton, 393.

- Stratham RR (1990) Why life histories evolve differently in the sea. *Am. Zool.* **30**, 197-207.
- Tallmon DA, Koyuk A, Luikart G, Beaumont MA (2008) Computer programs: Onesamp: a program to estimate effective population size using approximate Bayesian computation. *Mol Ecol Resources* **8**, 299-301.
- Teacher AGF, Griffiths DJ (2011) HapStar: automated haplotype network layout and visualization. *Mol Ecol Resources* **11**, 151-153.
- ASSRT (2007) Status review of Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus*). Report to the National Marine Fisheries Service, Northeast Regional Office. February 23, 2007. 174 pp.
- Therkildsen NO, Nielsen EE, Swain DP, Pedersen JS (2010) Large effective population size and temporal genetic stability in Atlantic cod (*Gadhus morhua*) in the southern Gulf of St. Lawrence. *CAN J FISH AQUAT SCI* **67**, 1585-1595.
- Thompson JD, Gibson TJ, Higgins DG (2002) Multiple sequence alignment using ClustalW and ClustalX. *Current Protocols in Bioinformatics* **2**, 1-22.
- Thorrold SR, Latkoczy C, Swart PK, Jones CM (2001) Natal homing in a marine fish metapopulation. *Science* **291**, 297-299.
- Todorov JR, Elskus AA, Schlenk D, Ferguson PL, Brownawell BJ (2001) Estrogenic responses of larval sunshine bass (*Morone saxatilis* x *M. chrysops*) exposed to New York City sewage effluent. *Mar Environ Res* **54**, 691-695.
- Towner AV, Weisel MA, Reisinger RR, Edwards D, Jewell OJ (2013) Gauging the threat: The first population estimate for White Sharks in South Africa using photo identification and automated software. *PLOS One* **8**, e66035.
- Turner TF, Osborne MJ, Moyer GR, Benavides MA, Alo D (2006) Life history and environmental variation interact to determine effective population to census size ratio. *Proc Roy Soc B* **273**, 3065-3073.
- Turner TF, Wares JP, Gold JR (2002) Genetic effective size is three orders of magnitude smaller than adult size in an abundant, estuarine-dependent marine fish (*Sciaenops ocellatus*). *Genetics* **162**, 1329-1339.
- van Guelpen L, Davis CC (1979) Seasonal movements of the Winter Flounder, *Pseudopleuronectes americanus*, in two contrasting inshore locations in Newfoundland. *TAFS* **108**, 26-37.
- Verissimo A, McDowell JR, Graves JE (2010) Global population structure of the spiny dogfish *Squalus acanthias*, a temperate shark with an antitropical distribution. *Mol Ecol* **19**, 1651-1662.
- Verissimo A, McDowell JR, Graves JE (2011) Population structure of a deep-water squaloid shark, the Portuguese dogfish (*Centroscymnus coelolepis*). *ICES Journal of Marine Science* **68**, 555-563.
- Wahlund S (1928) Zusammensetzung und Population und Korrelationserscheinung vom Standpunkt der Vererbungslehre aus betrachtet. *Hereditas* **11**, 65-106.
- Waldman JR, Hart JT, Wirgin II (1996) Stock composition of the New York Bight Atlantic sturgeon fishery based on analysis of mitochondrial DNA. *TAFS* **125**, 364-371.
- Waldman JR, King T, Savoy T, Maceda L, Grunwald C, Wirgin I (2012) Stock origins of subadult and adult Atlantic Sturgeon, *Acipenser oxyrinchus*, in a non-natal estuary, Long Island Sound. *Estuar Coasts* doi 10.1007/s12237-012-9573-0.

- Waldman JR, Wirgin II (1998) Status and restoration options for Atlantic sturgeon in North America. *Conserv Biol* **12**, 631-638.
- Wang C, Yu X, Tong J (2007) Microsatellite diversity and population genetic structure of redfin culter (*Culter erythropterus*) in fragmented lakes of the Yangtze River. *Hydrobiologica* **586**, 321-329.
- Wang J (2001) A pseudo-likelihood method for estimating effective population size from temporally spaced samples. *Genetical Research* **78**, 243-257.
- Wang J, Whitlock MC (2003) Estimating effective population size and migration rates from genetic samples over space and time. *Genetics Soc. America* **163**, 429-446.
- Waples RS (1989) A generalized approach for estimating effective population size from temporal changes in allele frequency. *Genetics* **121**, 379-391.
- Waples RS (1990) Conserv Gen of Pacific salmon. II. Effective population size and the rate of loss of genetic variability. *J Her* **81**, 267-276
- Waples RS (1990a) Conserv Gen of Pacific salmon. III. Estimating effective population size. *J Her* **81**, 277-289.
- Waples RS (2005) Genetic estimates of contemporary effective population size, to what time periods do the estimates apply?. *Mol Ecol* **14**, 3335-3352.
- Waples RS (2006) A bias correction for estimates of effective population size based on linkage disequilibrium at unlinked gene loci. *Conserv Gen* **7**, 167-184.
- Waples RS, Do C (2008) LDNe: a program for estimating effective population size from data on linkage disequilibrium. *Mol Ecol Resources* **8**, 753-756.
- Waples RS, Do C (2009) Linkage disequilibrium estimates of contemporary Ne using highly variable genetic markers: a largely untapped resource for applied conservation and evolution. *Evolutionary applications*, 10.1111/j.1752-4571.2009.00104.x.
- Waples RS, Teel DJ. (1990b) Conserv Gen of Pacific salmon I. Temporal changes in allele frequency. *Conserv Biol* **4**, 144-156.
- Ward RD, Woodward M, Skibinski DOF (1994) A comparison of genetic diversity levels in marine, freshwater, and anadromous fishes. *J Fish Biol* **44**, 213-232.
- Watts PC, Kay SM, Wolfenden D, Fox CJ, Geffen AJ, Kemp SJ, Nash RDM (2009) Temporal patterns of spatial genetic structure and effective population size in European plaice (*Pleuronectes platessa*) along the west coast of Scotland and in the Irish Sea. *ICES Journal of Marine Science* **67**, 607-616.
- Watts PC, Nash RDM, Kemp SJ (2004) Genetic structure of juvenile plaice *Pleuronectes platessa* on nursery grounds within the Irish Sea. *Journal of Sea Research* **51**, 191-197.
- White TA, Fortherby HA, Stephens PA, Hoelzel AR (2011) Genetic panmixia and demographic dependence across the North Atlantic in the deep-sea fish, blue hake (*Antimora rostrata*).
- White TA, Steffani S, Stamford J, Hoelzel AR (2009) Unexpected panmixia in a long-lived, deep-sea fish with well-defined spawning habitat and relatively low fecundity. *Mol Ecol* **18**, 2563-2573.
- Wiehe T (1998) The effect of selective sweeps on the variance of the allele distribution of a linked multiallele locus: Hitchhiking of microsatellites. *Theoretical Population Biology* **53**, 272-283.

- Willi Y, van Buskrik J, Hoffmann AA (2006) Limits to the adaptive potential of small populations. *Ann Rev Ecol Evol Syst* **37**, 433-458.
- Williamson-Natesan EG (2005) Comparison of methods for detecting bottlenecks from microsatellite loci. *Conserv Gen* **6**, 551-562.
- Wilson EO, Brown Jr. WL (1953) The subspecies concept and its taxonomic application. *System Zool* **2**, 97-111.
- Wirgin I, Maceda L, Waldman JR, Wehrell Sm Dadswell M, King T (2012) Stock origin of migratory Atlantic sturgeon in Minas Basin, Inner Bay of Fundy, Canada, determined by microsatellite and mitochondrial DNA Analyses. *TAFS* **141**, 1389-1398.
- Wirgin I, Waldman JR, Rosko J, Gross R, Collins MR, Rogers SG, Stabile J (2000) Genetic structure of Atlantic Sturgeon populations based on mitochondrial DNA control region sequences. *TAFS* **129**, 476-486.
- Wirgin I, Waldman JR, Stabile J, Lubinski B, King T (2002) Comparison of mitochondrial DNA control region sequence and microsatellite DNA analysis in estimating population structure and gene flow rates in Atlantic sturgeon *Acipenser oxyrinchus*. *J. Appl. Ichtyol.* **18**, 313-319.
- Wright S (1922) Coefficients of inbreeding and relationship. *The American Naturalist* **56**, 330-338.
- Wright S (1931) Evolution in Mendelian populations. *Genetics* **16**, 97-159.
- Wright S (1965) The interpretation of population structure by F-statistics with special regard to systems of mating. *Evolution*, 395-420.
- Wright S (1984) Evolution and the genetics of populations. *Chicago: University of Chicago Press*.
- Wuenschel MJ, Ablel KW, Byrne D (2009) Seasonal patterns of winter flounder *Pseudopleuronectes americanus* abundance and reproductive condition on the New York Bight continental shelf. *J Fish Biol* **74**, 1508-1524.
- Yencho MA (2009) Abundance, mortality, age and growth of young of the year winter flounder (*Pseudopleuronectes americanus*) in two locations on Long Island. *Master's Thesis*.
- Zarraonaindia I, Pardo MA, Iriondo M, Manzano C, Estonba A (2009) Microsatellite variability in European anchovy (*Engraulis encrasicolus*) calls for further investigation of its genetic structure and biogeography. *International council for the exploration of the sea (ICES)*.

Appendix A

Molecular and morphometric assessment of the taxonomic status of *Menidia conchorum*

(to be submitted to Bulletin of Marine Science)

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Abstract

On the continuum of speciation an ecotype defines a subdivision below the species level adapted to a specific set of environmental conditions found in a distinct habitat. Ecotypes are distinguished by strong morphological, genetic, behavioral and physiological differences compared to populations of the same species inhabiting other habitats. In the coastal waters of southern Florida, three species of *Menidia* (Family Atherinopsidae) have traditionally been distinguished: *Menidia beryllina*, the inland silverside, found in coastal brackish habitats, *Menidia peninsulae*, the tidewater silverside which inhabits the coastal waters and *Menidia conchorum* which is endemic to the Florida Keys and inhabits tidally restricted hypersaline pools. However, due to a lack of diagnostic morphological characteristics separating them *M. conchorum* may be an ecotype of *M. peninsulae*. This taxonomic uncertainty has hindered conservation efforts for *M. conchorum*. Here we used a combination of phylogenetics and morphometric methods to address the taxonomic status of *M. conchorum*. We used 17 landmarks to compare the morphology of *M. peninsulae* and *M. conchorum* and performed phylogenetic reconstruction using maximum parsimony and maximum likelihood methods for one nuclear

(ITS2) and one mitochondrial locus (ND2). Both mitochondrial and nuclear phylogenetic reconstructions indicated that *M. conchorum* and *M. peninsulae* are not reciprocally monophyletic and hence do not meet the criteria of the phylogenetic species concept. However, we found significant morphometric differences between the two nominal species and determined different haplotype frequencies for *M. conchorum* and *M. peninsulae*. Thus, *M. conchorum* is best classified as a genetically distinct ecotype of this widely distributed species.

Introduction

On the continuum of speciation an ecotype defines a subdivision below the species level adapted to a specific set of environmental conditions (Begon 2006) found in a unique habitat. Similar to a subspecies or race it is distinguished by strong morphological, genetic, behavioral and physiological differences but is set apart by being clearly defined by the specific environment it inhabits (Wilson & Brown 1953). Reciprocal monophyly at many independent loci is widely regarded as the Key criteria for species delineation under the phylogenetic species concept (Cracraft 1989, de Queiroz 2007) though it does not emphasize reproductive isolation as a delineating criterion, which is the fundamental component of the biological species concept (Mayr 1963, Avise & Ball 1990). Ecotypes, however, are not necessarily reciprocally monophyletic with their parent species and can successfully interbreed with them in hybrid zones. Ecotypes are considered to be a potential precursory step towards speciation (Begon 2006).

The New World Silversides (Atherinopsidae) encompass marine, estuarine and freshwater fishes with an elongated, silver-colored body and ranging in size from 2- 40cm, though most are smaller than 15cm. They share life-history characteristics of being short-lived, broadcast spawners and a short generation time (Allen 1998, Nelson 2006). Marine silversides inhabit coastal areas including estuaries and coastal lagoons and often form large schools (Bamber & Henderson 1988, Beheregaray & Levy 2000, Bloom et al. 2009). They have a broad geographic range with a continuous distribution and individual populations are influenced by local selection pressures (Beheregaray & Levy 2000, Beheregaray & Sunnucks 2001). Atheriniformes are characterized by high levels of intraspecific morphological variation due to high phenotypic plasticity, which allows them to adapt to a wide range of environments (Gosline 1948, Edwards et al. 1978, Chernoff 1982, Bamber & Henderson 1988). As a result the taxonomy of closely related species is often unresolved and the question of species validity of

distinct morphs inhabiting distinct habitats is common (Gosline 1948, Bloom et al. 2009, Fluker et al. 2011). Silversides are known to exhibit large variations in traits like body size, vertebral number and fin rays along latitudinal or salinity gradients, as a consequence of phenotypic plasticity and genotype x environment interactions (Billerbeck, et al. 1997, Yamahira et al. 2006). Changes in morphology along habitat (freshwater to marine) and latitudinal gradients can therefore confound the taxonomic resolution of closely related silverside species (Yamahira & Conover 2002, Yamahira et al. 2006, Fluker et al. 2011).

The genus *Menidia* consists of 7 - 8 species found along the Atlantic Coast and the Gulf of Mexico, from Maine to Veracruz, Mexico and in the Mississippi drainage and the Florida Keys (Gilbert & Lee 1980, Bloom et al. 2009). Southern Florida is home to three *Menidia* species, *Menidia beryllina*, the inland silverside, found in coastal brackish habitats, *Menidia peninsulae*, the tidewater silverside which inhabits the coastal waters and *Menidia conchorum* which is endemic to the Florida Keys and inhabits inland hypersaline pools that receive limited tidal exchange (Getter 1981). During extensive sampling from 1974-1975, Getter (1981) found Key silversides in 20 hypersaline lagoons and ponds and suggested that the species was in danger of extinction. In a follow-up survey, Key silversides were found at fewer (15) sites, suggesting that that they may be vulnerable to extirpation because the hypersaline ponds and lagoons tend to be small, few in number, and subject to development when found on private property (Conover et al. 2000). Key silversides has at times been classified as “endangered” (Florida Committee on Rare and Endangered Plants and Animals), “globally imperiled” (Florida Natural Areas Inventory) or “threatened” (Florida Fish and Wildlife Conservation Commission). The National Marine Fisheries Service (NMFS) identified the Key silverside as a candidate species for listing under the Endangered Species Act (ESA) in 1991 (56 FR 26797) and transferred the species to the Species of Concern list in 2004 (69 FR 19975). Species of Concern are those species about which NMFS has some concerns regarding status and threats, but for which insufficient information is available to indicate a need to list the species under the ESA.

As is true of some other silversides, the taxonomic status of *M. conchorum* is the subject of debate, with the Key question being whether it is a distinct species as opposed to being an ecotype of *M. peninsulae* (Duggins et al. 1986, Conover et al. 2000). It has been questioned whether the meristic differences used to separate the two species actually represent different points on a latitudinal cline given that there are no morphological features that are clearly

diagnostic for the two species (Duggins et al. 1986, Conover et al. 2000; Yamahira et al. 2006). While *M. beryllina* is reciprocally monophyletic with *M. peninsulae* and is clearly a valid species (Johnson 1975, Duggins et al. 1986, Bloom et al. 2009) the phylogenetic status of *M. conchorum* and *M. peninsulae* remain unclear. Early analysis of allozyme variation indicated that *M. conchorum* was genetically similar to *M. peninsulae*. Similarly, a recent phylogenetic study show *M. peninsulae* and *M. conchorum* to be conspecific (Bloom et al. 2009), though this study only used 1-2 ND2 haplotypes per species looking to resolve the relationships of the entire silverside tribe Menidii and was concerned at establishing monophyly of the families contained within and did not focus on detailed relationships between individual species. In light of confounding evidence toward the taxonomic status of *M. conchorum*, this nominal species may actually be a genetically distinct ecotype of the widely distributed *M. peninsulae* but no prior research has tested this hypothesis.

Here we report an assessment of the taxonomic status of Key silversides using DNA sequence analysis and geometric morphometrics. Complementing the genetic tests, morphology provides a useful tool to compare divergence among groups. The rate of speciation or taxonomic divergence may differ from that of morphology, and the nature of these differences speaks to the mechanism of evolution and patterns of phenotypic selection among groups (Foote 1993). We further examine mitochondrial and nuclear phylogenetic reconstructions to test the hypothesis of Key and tidewater silversides being distinct species, predicting that they would be reciprocally monophyletic at all tested loci if this were the case. In addition, we compared the body shape of *M. conchorum* and *M. peninsulae* in order to test for the presence of morphological variation, hypothesizing that significant morphological differences exist even in absence of strict genetic divergence.

Methods

Sample collection

Tissue samples were collected from several locations in southern Florida for genetic and morphometric analysis (Figure A-1). For phylogenetic reconstruction we used archived *M. conchorum* from two Florida Key locations (Sugarloaf Key (SK), Grassy Key (GK)) and *M. beryllina* (Crocodile Lakes (CL)) originally obtained by Conover et al. (2000). In addition, *M. peninsulae* samples were obtained from trawls performed by the Florida Fish and Wildlife Conservation Commission (FWC) in the Gulf of Mexico (Charlotte Harbor (CH)), detailed gear

description and sampling protocols can be found in Poulakis et al. 2003. The same *M. peninsulae* samples were also used for morphometric analysis. Because the originally utilized archives samples of *M. conchorum* were of too poor quality to use for morphometric analysis, in February 2012, nine Florida Key sample sites (Conover et al. 2002) were revisited and sampled (Figure A-1). Additional *M. peninsulae* samples for morphometric comparison were obtained from a second Gulf location (Sarasota Bay (SB)). Upon capture, fish were quickly frozen and preserved as such until imaging. This allowed them to retain their pliability for standardized positioning.

DNA extraction and PCR amplification

Phylogenetic reconstruction was performed using *M. conchorum* tissue samples from two Florida Key locations (SK, N=21, GK, N=31), *M. peninsulae* from one Gulf location (CH, N=21), and *M. beryllina* from one Florida Key location (Figure A-1). DNA was extracted using 15 – 30 mg of tissue using a standard commercial protocol (DNeasy, Qiagen, Valencia, California). We amplified one mitochondrial locus, NADH dehydrogenase2 (ND2) and one nuclear locus, Internal transcription spacer2 (ITS2). ND2 and ITS2 are commonly used to delineate species. *Menidia beryllina* was included in our phylogenetic analysis as a known monophyletic group to offer context of genetic divergence and to root out trees.

Primers for the mitochondrial loci were designed using sequences of the mitochondrial genome of *Menidia menidia* (Accession number: AB370893.1). To design primers for the internal transcription spacer 2 (ITS2), we initially used universal primers (Plank et al. 2010) and then designed internal primers by eye from the sequences we obtained. All loci were PCR amplified in 50 μ L volumes containing genomic DNA, 1X PCR buffer (Qiagen Inc., Valencia, California), 40 μ M dNTPs, 12.5 μ mol of each of the primers. Thermal cycling was conducted in a Labnet Multigene TC9600-G thermocycler for 35 cycles of 1 min at 95°C, 1 min at the primer-specific annealing temperature T_a (Table A-1) followed by a final extension step of 72°C for 10 min. PCR products were purified using ExonucleaseI and Shrimp Alkaline Phosphatase according to the manufacturer's protocol (Affymetrix, Santa Clara, California). Sequencing was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, California) in a Bio-RAD Dyad thermocycler for 25 cycles of 96°C for 10s, 50°C for 5s, and 60°C for 4 min. Sequencing reactions were precipitated with ethanol and 125mM EDTA and run on an ABI 3730 DNA.

Phylogenetic analysis

Sequences were validated by eye using Chromas 2.33 (<http://www.technelysium.com.au>). All distinct sequences were verified by sequencing them in both the forward and reverse direction. These sequences were then individually checked for quality, trimmed, edited by eye and then imported into ClustalX (Thompson JD 2002) for alignment. The alignment was exported into ARLEQUIN v3.5 (Excoffier L 2010) to identify and characterize haplotypes using the Tamura & Nei model.

Phylogenetic reconstruction was conducted using Maximum-Likelihood (ML) and maximum parsimony (MP) analysis. The best model of nucleotide substitution for each gene was determined using the model test implemented in MEGA 5.1 (Tamura et al. 2011) which compares 24 substitution models. The best fit model for ITS2 was the Tamura 3-parameter model (Tamura 1992), for ND2 the Kimura 2-parameter model. For ML analysis of both genes evolutionary rate differences among sites were best modeled through a gamma distribution using 6 discrete gamma categories. MP and ML were conducted using MEGA5.1 (Tamura et al. 2011), bootstrap probabilities were determined using 1000 replicates (Felsenstein 1985). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei 2000) with search level one in which the initial trees were obtained through the random addition of sequences (10 replicates). Up to 100 most parsimonious trees were retained. For the ML analysis initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The topology with the superior log likelihood value was selected.

Geometric morphometrics

For morphometric comparison, we combined *M. peninsulae* individuals from two Gulf locations (N=65) and *M. conchorum* from 9 Florida Key pools (N=238) to achieve adequate sample sizes. In order to reduce a potential source of bias relating to allometry, we only included individuals within the overlapping centroid size range in both groups. We placed specimens with their right lateral sides facing upward and photographed them with a digital camera (Fujifilm Finepix S4500) set on a tripod. Next, a shape file was created with the program tpsUtil (Rohl 2012) and used tpsDIG2 (Rohl 2010) to digitize 17 landmarks on each individual (Figure A-2) following Fluker et al. (2011). Once digitized, shape variables were derived via generalized Procrustes analysis (GPA) in MorphoJ (Klingenberg 2011). GPA is a generalized least-squares

shape fitting procedure that aligns specimens by scaling, rotating and translating their shapes, as defined by homologous landmarks (Zelditch et al. 2004).

Discriminate function analysis (DFA) embedded in MorphoJ (Klingenberg 2011) was used to identify shape differences among *Menidia* species. Shape disparities were visualized using the thin-plate spline transformation (Bookstein 1991). We performed a multivariate regression of shape on centroid size to test for allometric effects and compared the responses among *Menidia* groups with a MANCOVA in tpsRegr (Rohlf 2011). Lastly, we created a categorical independent factor by dividing the covariate, centroid size, into three groups (i.e. small, medium and large) with equal ranges. We performed additional DFAs comparing *M. conchorum* and *M. peninsulae* in each size group to verify that the overall direction of shape change was consistent across sizes of fish analyzed.

Results

Haplotype analysis and phylogenetic reconstruction

Thirty-eight individuals were sequenced for a 267bp fragment of the ITS2 gene containing 31 polymorphic sites. Haplotype analysis revealed one shared haplotypes between *M. conchorum* and *M. peninsulae*, and *M. conchorum* was determined to be less diverse than *M. peninsulae* at this locus, with eight (n = 21) distinct haplotypes compared to 19 *M. peninsulae* haplotypes (n=45). For *M. beryllina* five distinct haplotypes were found. Phylogenetic reconstruction of ITS2 indicated *M. beryllina* to be reciprocally monophyletic for both ML and MP analysis (Figs A-3, A-4), whereas *M. conchorum* and *M. peninsulae* were found to be polyphyletic.

A similar pattern was detected using the mitochondrial phylogenetic reconstruction of 252bp of the mtND2 gene sequence containing 27 polymorphic sites sequenced in 49 individuals. We identified four *M. beryllina* haplotypes, 19 *M. peninsulae* haplotypes and six *M. conchorum* haplotypes, two of which were shared. Again, both MP and ML analysis confirm *M. beryllina* as reciprocally monophyletic with *M. conchorum* and *M. peninsulae*, whereas the latter two were polyphyletic (Figures A-5, A-6).

General observations of both sequenced loci are that *M. beryllina* forms a reciprocally monophyletic group while *M. peninsulae* and *M. conchorum* are polyphyletic. At times, a single *M. conchorum* haplotype is found within a *M. peninsulae* clade and vice versa, indicative of incomplete lineage sorting. Bootstrap values supporting the *M. conchorum* and *M. peninsulae*

clades are generally low, pointing towards the uncertainty of the taxonomic relationship of the haplotypes. In addition, *M. peninsulae* generally has a higher genetic diversity, with more unique haplotypes identified than *M. conchorum*.

Geometric morphometrics

Hotelling's T^2 test on Procrustes distances from 10,000 DFA permutations indicated that the shape of *M. conchorum* was significantly distinguishable ($p < 0.0001$) from that of *M. peninsulae* (Figure A-7). Shape changes among groups were characterized by dorso-ventral deepening of the midsection and a more robust head in *M. conchorum* relative to *M. peninsulae*, resulting in an overall stouter appearance in the former (Figure A-7b). Additionally, there was a significant relationship between centroid size and shape (multivariate regression, $p < 0.0001$ on 10,000 permutations), but the rate of shape change was marginally, although significantly, different among silverside groups (MANCOVA, $p = 0.044$). Because the slopes of individual groups were not homogenous, we could not use a single function to remove the effects of size. DFAs based on 10,000 permutations, showed that there was significant discrimination among silverside groups in each size class ($n_{\text{small}} = 123$, $p_{\text{small}} < 0.0001$; $n_{\text{medium}} = 113$, $p_{\text{medium}} < 0.0001$ and $n_{\text{large}} = 67$, $p_{\text{large}} = 0.0001$). Further, visualization of mean shapes for each group confirmed that the direction of change within size classes (Figure A-8) was consistent with overall shape variation shape variation between *M. conchorum* and *M. peninsulae* (Figure A-7b). Hence, the observed shape differences likely reflected true variation between silverside groups, not artifacts of size differences among samples.

Discussion

The pattern of genetic and morphometric variation within and among these two nominal species found in our study is differentiated between the two corresponding habitats of the tidally restricted hypersaline pools containing *M. conchorum* and the coastal marine habitat of *M. peninsulae*. Phylogenetic trees based on both mitochondrial and nuclear DNA indicate significant genetic divergence, though full reciprocal monophyly is yet to occur. The complementary analysis of geometric morphometrics demonstrates corresponding differences in body shape. Marked differences in haplotype frequencies and morphometric analysis between these two groups indicate that *M. conchorum* is not a valid species but instead represents an ecotype of the more widely distributed *M. peninsulae*.

Phylogenetic reconstruction further confirms *M. beryllina* as a valid species (Johnson 1975, Bloom et al. 2009), yet we reject the hypothesis that *M. conchorum* forms a reciprocally monophyletic group distinct from *M. peninsulae*. According to the phylogenetic species concept, these two forms should thus not be regarded as distinct species. Bootstrap values supporting the clades of *M. conchorum* and *M. peninsulae* were low for both ND2 and ITS2, further demonstrating the uncertainty of the taxonomy of these two species. Single samples of *M. conchorum* found in *M. peninsulae* clades are indicative of either recent gene flow or insufficient time for complete sorting. Given the fragmented habitat of *M. conchorum* contemporary gene flow is unlikely, though the time span for which these hypersaline pools have been cut off from the open ocean is unclear. The Florida Keys are estimated to be about 10,000 years old (Hoffmeister & Multer 1968), a time span which may not be sufficient for complete lineage sorting.

Consistent morphological differences between *M. conchorum* and *M. peninsulae* were detected, but the reason for the disparity between genetic and morphological data remains unknown. Silverside fishes along the North American east coast (*M. menidia*, *M. peninsulae*) have been found to display countergradient growth variation, where individuals grow faster at higher latitudes to compensate for shorter growing seasons (Conover & Present 1990, Yamahira & Conover 2002, Yamahira et al. 2007). Importantly, these results indicate that size selection is driving the relationship, thus different growth rates have a genotypic component. In each size group that we compared, *M. peninsulae* (which was sampled at a higher latitude than *M. conchorum*) had more robust, fusiform body shapes (Figure A-8). It is possible that morphological differences among groups in this study are partially due to differences in growth rates, as in *M. menidia*. However, if *M. conchorum* and *M. peninsulae* represent two populations of the same species whose mixing has become limited, it is likely that morphological differences are the result of both population divergence due to isolation and to selection on growth rates along an environmental gradient.

Our results show that the *M. conchorum* may have originated after inland incursions by *M. peninsulae* which resulted in landlocked populations. *Menidia peninsulae* exhibited higher diversity at all loci than *M. conchorum* consistent with their wider geographic distribution and larger population size. Multiple studies have suggested that fish species invading a new habitat thus being faced with a shift in ecological conditions and selective pressures may experience a

rapid adaptive divergence resulting in a reduction of gene flow, ultimately leading to speciation (Cracraft 1982, Beheregaray & Sunnucks 2001, Barluenga et al. 2006, Fluker et al. 2011, Bloom et al. 2013). Ecotypes form a precursory step in this process, fulfilling neither the biological nor the phylogenetic species concept yet possessing distinct differences in morphology, behavior and physiology and landing them on the brink of speciation (e.g. Barbour 1973, Beheregaray & Levy 2000, Beheregaray & Sunnucks 2001, Trabelsi et al. 2004, Klossa-Kilia et al. 2007, Fluker et al. 2011). In the case of *M. conchorum* the invasion of a new habitat has resulted in a shift of environmental, in particular salinity. Salinity has previously been suggested to play a role in the segregation and speciation of Menidia species (e.g. Gosline 1948; Johnson 1975; Fluker 2011). For example, *M. beryllina* and *M. peninsulae* have been shown to segregate based on salinity in coastal bays in which they are allopatric (Suttkus 1998), though their habitat may overlap in intermediate salinity ranges where they can successfully hybridize (Middaugh 1986; Middaugh 1987). Though we cannot make a statement about the force of selection acting here, Bamber and Henderson (1988) have proposed in model of speciation for silversides that when a new habitat is invaded it is individuals with a generalist genotype with the ability to adapt to a wide range of environmental conditions and habitat-specific selective pressures that are successful and form the founding group. This effect results in adaptive divergence and the emergence of ecotypes.

Currently, *M. conchorum* is listed as a Species of Concern by the National Marine Fisheries Service, mainly due to the threat of habitat degradation as the ongoing existence of *M. conchorum* strongly depends on the persistence of the hypersaline pools in the lower Florida Keys that are threatened by potential sea level rise and development projects in the Keys (Duggins et al. 1986, Conover et al. 2000). The resolution of the taxonomic status of *M. conchorum* has direct management and conservation implications (Conover et al. 2000). As an ecotype of *M. peninsulae*, *M. conchorum* forms an evolutionary significant unit that warrants independent management, which is best achieved by protecting the hypersaline pools they occupy. Further research into their life history, behavior, habitat requirements and ecology is necessary, coupled with continued monitoring of population trends.

We conclude that *M. conchorum* is an ecotype of *M. peninsulae* and recommend that even though these two forms are not separate species, they are genetically and morphometrically distinct and should therefore be protected and managed as separate units. These two forms do not co-occur and *M. conchorum* might play a vital role in the hypersaline pond ecosystem. Since *M.*

conchorum inhabits pools with very restricted access to the open ocean it is unlikely that *M. peninsulae* would re-colonize this unique habitat in the event that *M. conchorum* becomes extinct.

References

- Allen G (1998) in: Paxton, JR & Eschmeyer, WN, ed. Encyclopedia of Fishes. San Diego: Academic Press. pp 153-156.
- Awise JC, Ball RM (1990) Principles of genealogical concordance in species concepts and biological taxonomy. In: Futuyma, D., Antonovics, J., editors, *Oxford surveys in evolutionary biology*. New York: Oxford University Press. p. 45-67.
- Bamber RN, Henderson PA (1988) Pre-adaptive plasticity in atherinids and the estuarine seat of teleost evolution. *J Fish Biol* **33**, 17-23.
- Barbour CD (1973) A biogeographical history of *Chirostoma* (Pisces: Atherinidae): a species flock from the Mexican Plateau. *Copeia* **1973**, 533-556.
- Barluenga M, Stolting KN, Salzburger W, Muschick, M, Meyer A (2006) Sympatric speciation in Nicaraguan crater lake cichlid fish. *Nature* **439**, 719-723.
- Begon M (2006) Ecology: from individuals to ecosystems. *Blackwell Publishing 4th ed*, p. 5-8.
- Beheregaray LB, Levy JA (2000) Population genetics of the silverside *Odontesthes argentinensis* (Teleostei, Atherinopsidae): evidence for speciation in an estuary of southern Brazil. *Copeia* **2000**, 441-447.
- Beheregaray LB, Sunnucks P (2001) Fine-scale genetic structure, estuarine colonization and incipient speciation in the marine silverside fish *Odontesthes argentinensis*. *Mol Ecol* **10**, 2849-2866.
- Bloom DD, Pillar KR, Lyons J, Mercado-Silva N, Medina-Nava M (2009) Systematics and biogeography of the silverside tribe Menidii (Teleostomi: Atherinopsidae) based on the mitochondrial ND2 Gene. *Copeia* **2009**, 408-417.
- Bloom DD, Weir JT, Pillar KR, Lovejoy NR (2013) Do freshwater fishes diversify faster than marine fishes? A test using state-dependent diversification analyses and molecular phylogenetics of new world silversides (Atherinopsidae). *Evolution*.
- Bookstein FL (1991) Morphometric Tools for Landmark Data: geometry and Biology. *Cambridge University Press, New York, NY USA*. 435pp.

- Chernoff B (1982) Character variation among populations and the analysis of biogeography. *American Zoologist* **22**, 425-439.
- Conover D, Munch S, Lankford Jr. T (2000) Current status of the Key silverside, *Menidia conchorum*, in southern Florida Final Project Report. U.S. Geological Survey Biological Resources Division Restoration Ecology Branch, Florida Caribbean Science Center, Florida International University, Miami, FL 33199. 18p.
- Conover DO, Present TMC (1990) Countergradient variation growth rate: Compensation for length of the growing season among Atlantic silversides from different latitudes. *Oecologia* **83**, 316-324.
- Cracraft J (1982) A nonequilibrium theory for the rate-control of speciation and extinction and the origin of macroevolutionary patterns. *Syst Zool* **31**, 348-365.
- Cracraft J (1989) Speciation and its ontology: the empirical consequences of alternative species concepts for understanding patterns and processes of differentiation. In: Otte D., Endler, J., editors, Speciation and its consequences. Sunderland (MA): Sinauer. p. 28-59.
- de Queiroz K (2007) Species concepts and species delimitation. *Syst Biol* **56**, 879-886.
- Duggins Jr. C, Karlin A, Relyea K, Yerger R. (1986) Systematics of the Key silverside *Menidia conchorum* with comments on other *Menidia* species (Pisces Atherinidae). *Tulane Studies in Zoology and Botany* **25**, 133-150.
- Edwards RJ, Marsh E, Stevens Jr. FB (1978) The utility of the air bladder position in determining specific relationships in the atherinid genus *Menidia*. *Contrib Mar Sci* **21**, 1-7.
- Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Mol Ecol and Resources* **10**, 564-567.
- Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**.
- Fluker BJ, Pezold F, Minton RL (2011) Molecular and morphological divergence in the Inland silverside (*Menidia beryllina*) along a freshwater-estuarine interface. *Environ Biol Fish* **91**, 311-325.
- Foote M (1993) Disconcordance and concordance between morphological and taxonomic diversity. *Paleobiology* **19**, 185-204.
- Getter C (1981) Ecology and survival of the Key silverside, *Menidia conchorum*, an atherinid fish endemic to the Florida Keys. *PhD Dissertation. University of Miami*.

- Gilbert CR, Lee DS (1980) *Menidia beryllina*, p. 557. In: Atlas of North American Freshwater Fishes. D. S. Lee, C. R. Gilbert, C. H. Hocutt, R. E. Jenkins, D. E. McAllister, J. R. Stauffer (eds.). North Carolina State Museum of Natural History, Raleigh.
- Gosline WA (1948) Speciation in the fishes of the genus *Menidia*. *Evolution* **2**, 306-313.
- Herbert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identification through DNA barcodes. *Proc Roy Soc Lond B* **270**, 313-321.
- Hoffmeister JE, Multer HG (1968) Geology and Origin of the Florida Keys. *Geol Soc Am Bull* **79**, 1487-1502.
- Johnson WE (1975) Biochemical systematics of the atherinid genus *Menidia*. *Copeia* **4**, 662-691.
- Klingenberg CP (2011) MorphoJ: An integrated software package for geometric morphometrics. *Mol Ecol Resources* **11**, 353-357.
- Klossa-Kilia E, Papatropoulos V, Tryfonopoulos G, Alahiotis S, Kiliass G (2007) Phylogenetic relationships of the *Atherina hepsetus* and *atherina boyeri* (Pisces: Atherinidae) populations from Greece, based on mtDNA sequences. *Biol J Linn Soc* **92**, 151-161.
- Mayr E (1963) Animal species and evolution. Cambridge (MA): Harvard University Press.
- Middaugh DP, Hemmer MJ (1987) Reproductive ecology of the tidewater silverside, *Menidia peninsulae* (Pisces: Atherinidae) from Santa Rosa Island, Florida. *Copeia* **1987**, 727-732.
- Middaugh DP, Hemmer MJ, Rose YL (1986) Laboratory spawning cues in *Menidia beryllina* and *Menidia peninsulae* (Pisces: Atherinidae) with notes on survival and growth of larvae at different salinities. *Environ Biol Fish* **15**, 107-117.
- Nei M, Kumar S (2000) *Molecular Evolution and Phylogenetics*. Oxford University Press, New York.
- Nelson JS (2006) *Fishes of the world*, 4th edn. Wiley, Hoboken.
- Plank SM, Lowe CG, Feldheim KA, Wilson Jr. RR, Brusslan JA (2010) Population genetic structure of the round stingray *Urobatis halleri* (Elasmobranchii: Rajiformes) in southern California and the Gulf of California. *J Fish Biol* **77**, 329-340.
- Rohl FJ (2010) tpsDIG2 v2.16. <http://life.bio.sunysb.edu/morph/>. Accessed 17 February 2013.
- Rohl FJ (2011) tpsRegr v1.38. <http://life.bio.sunysb.edu/morph/>. Accessed 17 February 2013.
- Rohl FJ (2012) tpsUtil v1.53. <http://life.bio.sunysb.edu/morph/>. Accessed 17 February 2013.

- Suttkus RD, Metee MF (1998) Morphometric, meristic, and natural history notes on *Menidia beryllina* and *Menidia peninsulæ* in a marginal sympatric area in Perdido Bay, Alabama and Florida. *Southeast Fish Counc Proc* **37**, 7-13.
- Tamura K (1992) Estimation of number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. *Mol Biol Evol* **9**, 678-687.
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol Biol Evol* **28**, 2731-2739.
- Thompson JD, Gibson TJ, Higgins DG. (2002) Multiple sequence alignment using ClustalW and ClustalX. *Current Protocols in Bioinformatics* **2**, 1-22.
- Trabelsi M, Maamouri F, Quinard J-P, Boussard M, Faure E (2004) Morphometric or morpho-anatomical and genetic investigations highlight allopatric speciation in Western Mediterranean lagoons with the *Atherina lagunae* species (Teleostei, Atherinidae). *Estuar Coast Shelf S* **61**, 713-723.
- Wilson EO, Brown Jr. WL (1953) The subspecies concept and its taxonomic application. *System Zool* **2**, 97-111.
- Yamahira K, Conover DO (2002) Intra- vs. interspecific latitudinal variation in growth: adaptation to temperature or seasonality? *Ecol Evol* **83**, 1252-1262.
- Yamahira K, Lankford Jr. TE, Conover DO (2006) Intra- and interspecific latitudinal variation in vertebral number of *Menidia spp.* (Teleostei: Atherinopsidae). *Copeia* **3**, 431-436.
- Yamahira KM, Kawajiri M., Takeshi K, Irie T (2007) Inter- and intrapopulation variation in thermal reaction norms for growth rate: evolution of latitudinal compensation in ectotherms with a genetic constraint. *Evolution* **61**, 1577-1589.
- Zelditch ML, Swiderski DL, Sheets HD, Fink WL (2004) Geometric morphometrics for biologists: A primer. *Elsevier Academic Press, San Diego, CA, USA* 443pp.

Figure A-1: Sample locations for *Menidia* in southern Florida. Sample locations for *Menidia peninsulae* in the Gulf of Mexico (Sarasota Bay and Charlotte Harbor) and *Menidia conchorum* sampled in the Florida Keys. Numbers correspond to sample locations in Conover et al. 2000, abbreviations in parenthesis are used throughout this paper.

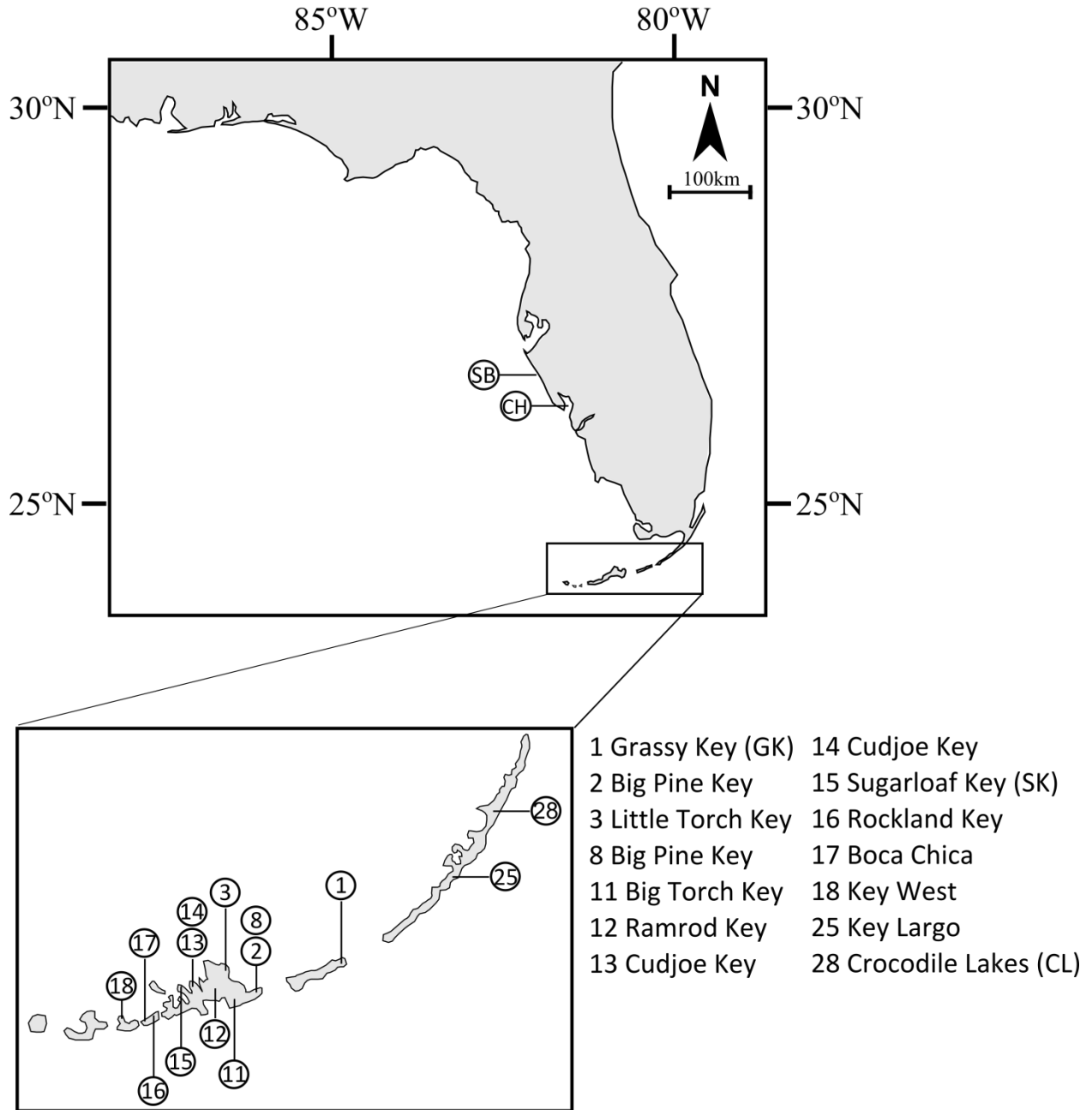


Figure A-2: Landmarks used for geometric morphometrics. Landmarks were chosen based on Fluker et al. 2011.

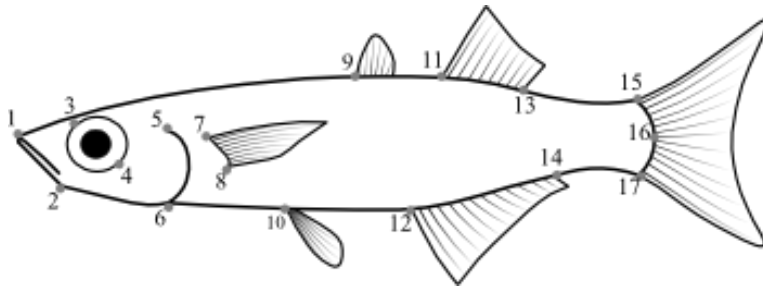


Figure A-3: Phylogenetic reconstruction of ITS2 gene (maximum parsimony). The evolutionary history was inferred using the maximum parsimony method based on a 267bp fragment of ITS2 with 31 polymorphic sites for haplotypes from *M. beryllina* (Mber), *M. peninsulae* (Mpen) and *M. conchorum* (Mcon). The absolute frequency of each haplotype is given in parenthesis. Tree #1 out of 9 most parsimonious trees (length = 48) is shown. The consistency index is 0.580645, the retention index is 0.861702, and the composite index is 0.628324 (0.500343) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated haplotypes clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

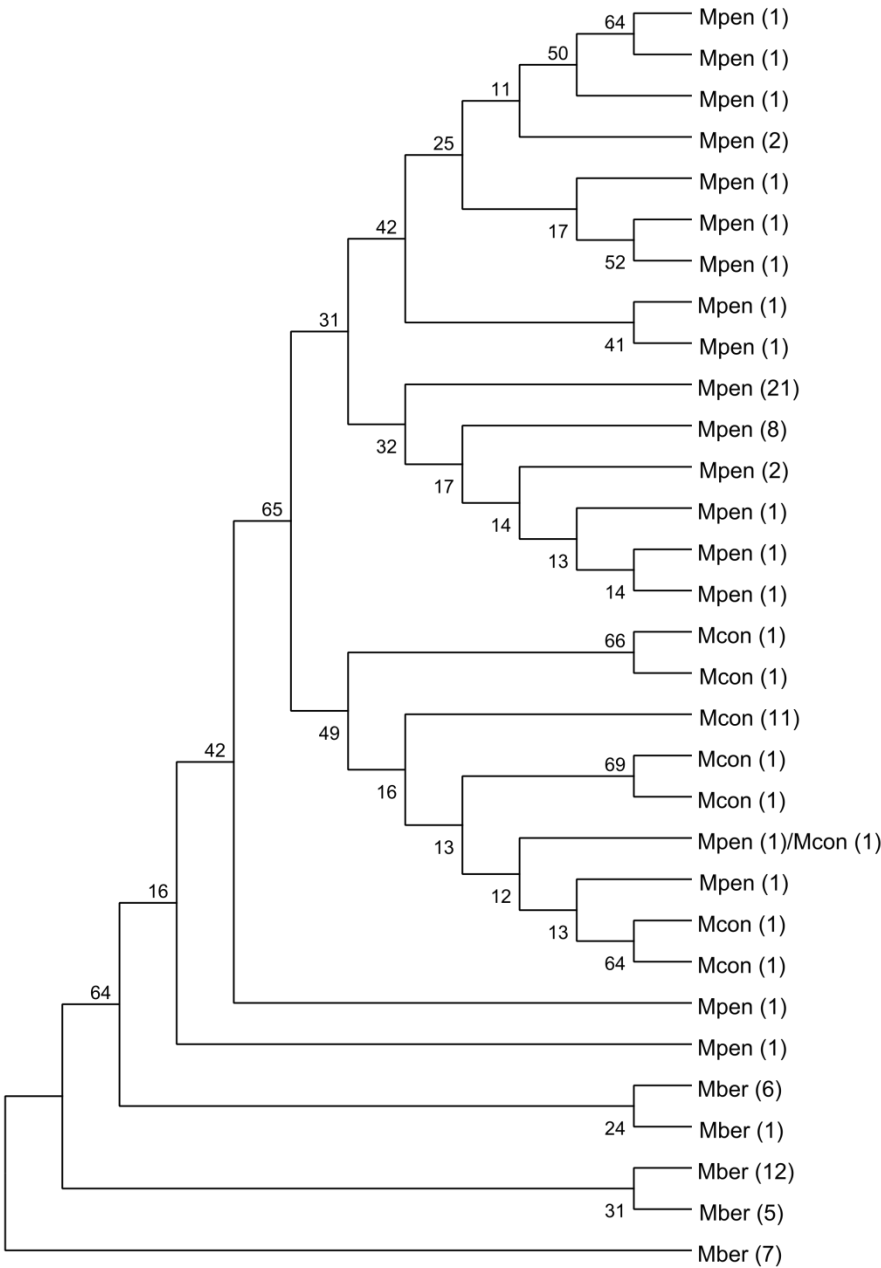


Figure A-4: Phylogenetic reconstruction of ITS2 gene (Maximum Likelihood). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura 1992) based on a 267bp fragment of ITS2 with 31 polymorphic sites for haplotypes from *M. beryllina* (Mber), *M. peninsulae* (Mpen) and *M. conchorum* (Mcon). The absolute frequency of each haplotype is given in parenthesis. The tree with the highest log likelihood (-643.7615) is shown. The percentage of trees in which the associated haplotypes clustered together is shown next to the branches. The tree is drawn to scale: branch lengths are measured in the number of substitutions per site.

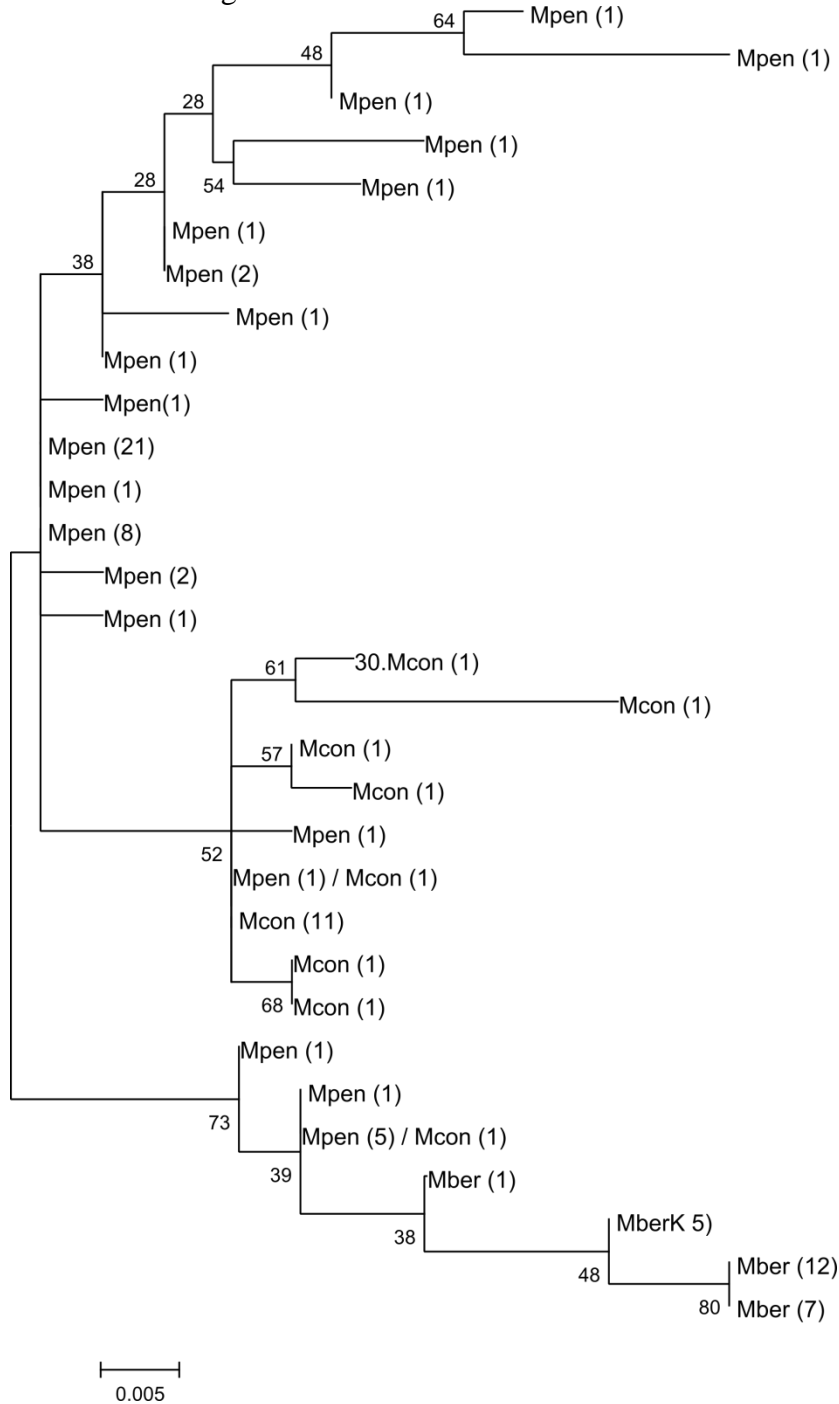


Figure A-5: Phylogenetic reconstruction of ND2 gene (maximum parsimony). The evolutionary history was inferred using the Maximum Parsimony method based on a 252bp fragment of ND2 with 27 polymorphic sites for haplotypes from *M. beryllina* (Mber), *M. peninsulae* (Mpen) and *M. conchorum* (Mcon). The absolute frequency of each haplotype is given in parenthesis. Tree #1 out of 8 most parsimonious trees (length = 54) is shown. The consistency index is (0.727273), the retention index is (0.900826), and the composite index is 0.700643 (0.655147) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated haplotypes clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

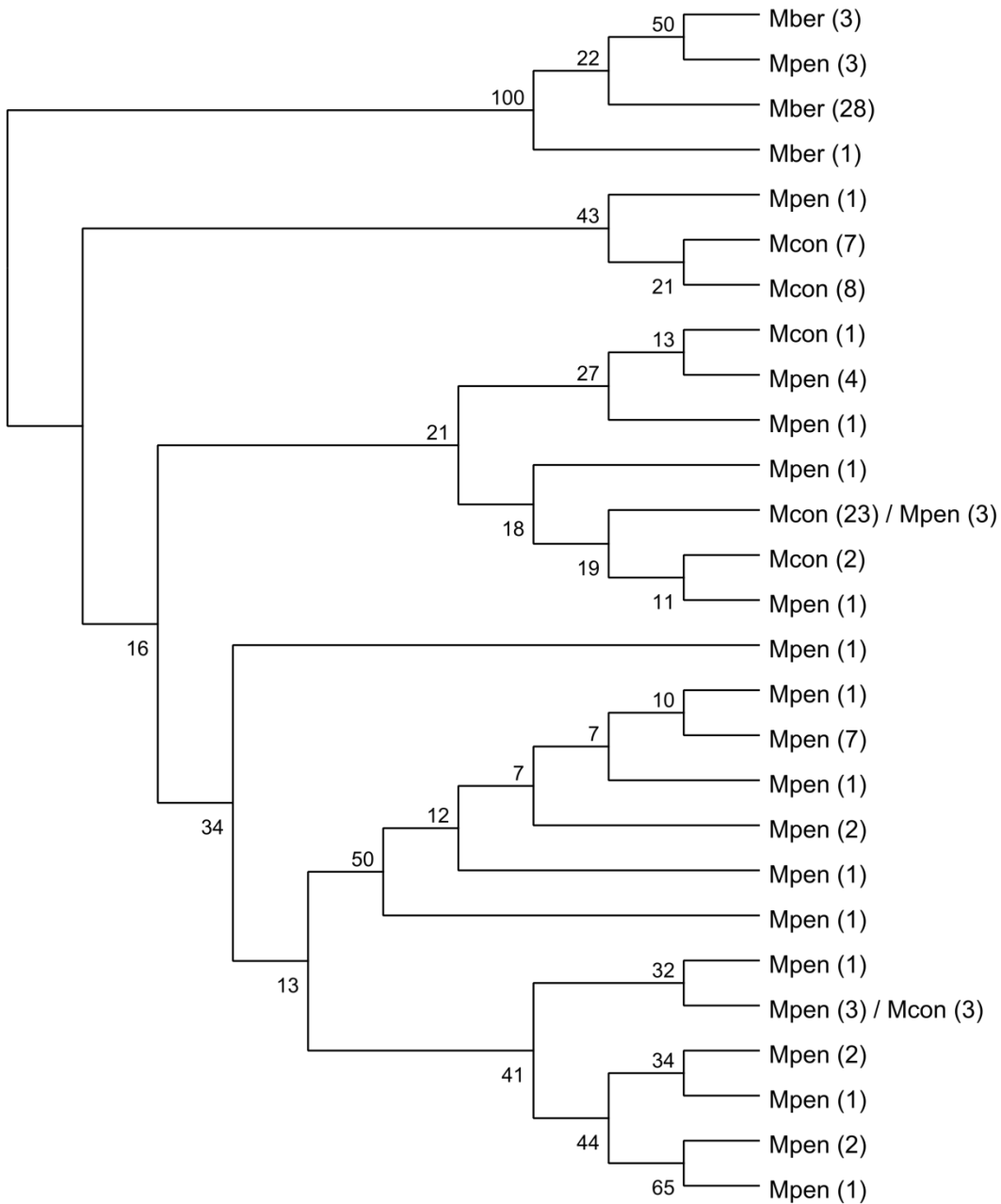


Figure A-6: Phylogenetic reconstruction of ND2 gene (maximum likelihood). The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model based on a 252bp fragment of ND2 with 27 polymorphic sites for haplotypes from *M. beryllina* (Mber), *M. peninsulae* (Mpen) and *M. conchorum* (Mcon). The absolute frequency of each haplotype is given in parenthesis. The tree with the highest log likelihood (-641.61) is shown. The percentage of trees in which the associated haplotypes clustered together is shown next to the branches. The tree is drawn to scale: branch lengths are measured in the number of substitutions per site.

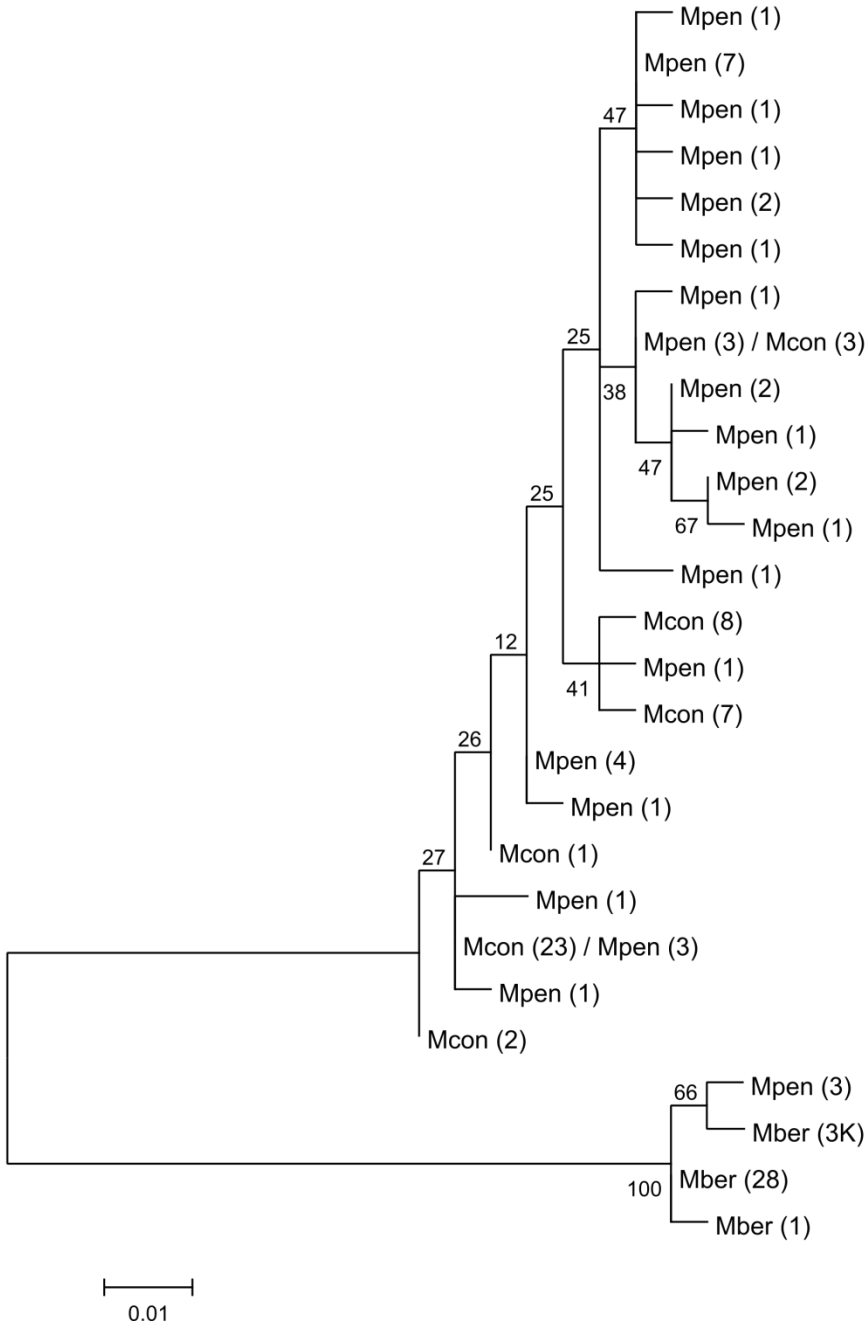
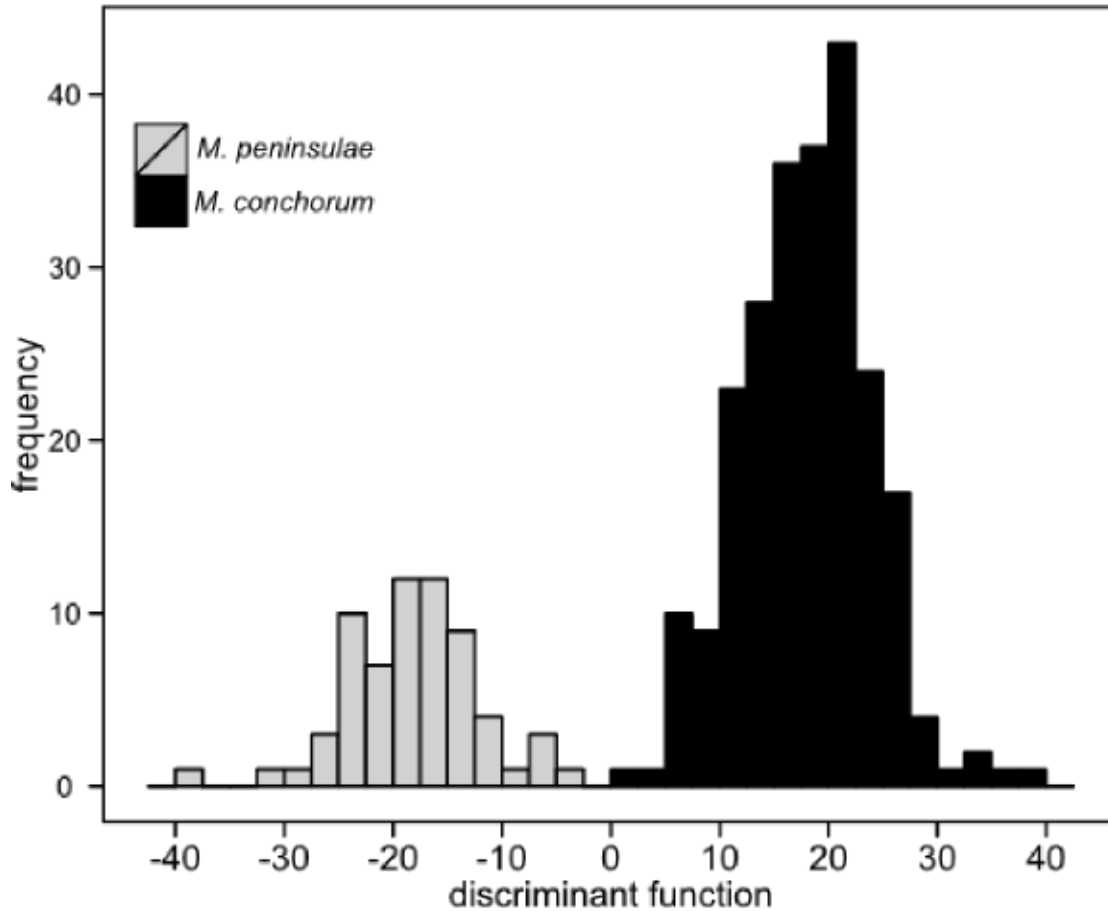


Figure A-7: Shape differences between *M. peninsulae* and *M. conchorum*. (A) Frequency of observations along a discriminant function defining shape differences between Gulf of Mexico (gray) and Florida Key (black) populations. (B) Mean shapes of Gulf of Mexico (gray line) and Florida Keys (black line) populations (scaled by a factor of two).

A



B

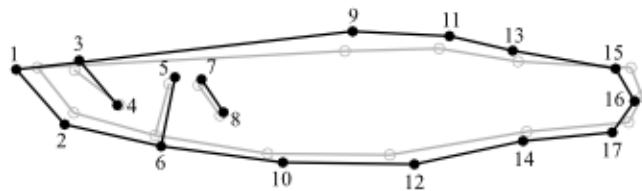


Figure A-8. Comparisons of mean shape of small medium and large fish among regions.
Shape differences were magnified by a factor of two to facilitate visualization.

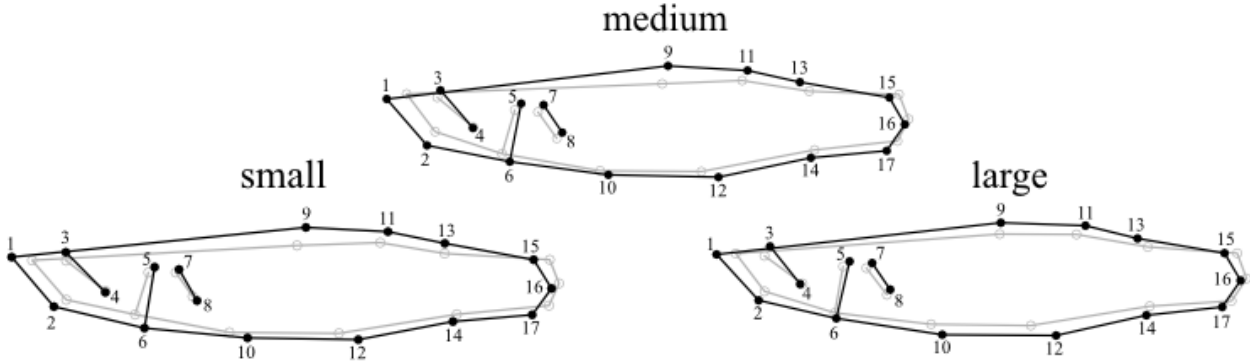


Table A-1: DNA sequences for phylogenetic analysis. Species-specific primers and annealing temperatures for ND2 and ITS2. Haplotype sequences have been deposited on Genbank.

locus	Primers	Ta [°C]	Accession No.
ND2	<i>MenND2F</i> :5'-TACTATAATTACCCTCGCCCTAGCC-3'	52	TBD
	<i>MenND2R</i> :5'-GTAGAGAAGGTTATGATGAAGTAGG-3'		
ITS2	<i>MenITS2F</i> :5'-GCAGGACACATTGATCATCGA-3'	55	TBD
	<i>MenITS2R</i> :5'-TCGGCAAGAGAGGGAGAGAC-3'		

Appendix B

List of publications

In Prep:

Title: DNA profiling reveals heightened vulnerability of white sharks, *Carcharodon carcharias*.

Authors:

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Summary:

Like many marine fish, it has proven very difficult to obtain information on the size and recent trajectory of white shark (*Carcharodon carcharias*) populations. We investigated patterns of genetic diversity in two regions: the Northwest Atlantic and southern Africa. We found these two populations to be genetically distinct at both mitochondrial and nuclear genetic loci and both exhibited signatures of recent population bottlenecks and contemporary inbreeding. The effective number of breeders in the Northwest Atlantic for the sampling period 2001-2008 was estimated to consist of approximately 21-49 individuals. These results highlight that genetic diversity assessment and genetics-based population size estimation can be a fruitful way to obtain information on the population size and trajectory of threatened marine fish that are otherwise difficult to assess.

Title: Genetic diversity and effective number of breeders of Atlantic sturgeon, *Acipenser oxyrinchus*.

Authors

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Keywords

Bottleneck, effective population size, mixed-stock analysis, individual-based assignment

Abstract

Juvenile Atlantic sturgeon (*Acipenser oxyrinchus*) forming aggregations at coastal sites in the mid-Atlantic Bight were subjected to a mixed stock analysis using 12 microsatellite loci. We confirmed earlier findings from analysis of mitochondrial DNA that three rivers (Hudson, James and Delaware River) are the main contributing spawning populations to these marine aggregations (~83%, 8% and 4%, respectively). We used these individuals to estimate the effective number of breeders (N_b) for each river during the time when these juveniles were spawned (Hudson River: 172-230; James: 40-100; Delaware: 75-186). The Hudson River estimate approximates one made using non-

genetic techniques, suggesting that the James and Delaware River estimates provide new information about the size of their spawning populations. We found evidence for a recent or ongoing bottleneck in all three rivers, with the Hudson being the least affected of the three (M-ratios Delaware = 0.57, Hudson = 0.68, James = 0.56) There were low but detectable levels of inbreeding in all three rivers. Genetic analysis of ocean-captured, juvenile sturgeon confirms that spawning populations are low and have suffered population bottlenecks severe enough to affect their contemporary genetic architecture.

Title: Inland incursion of a widespread marine fish leads to highly fragmented population structure and rapidly erodes genetic diversity.

Authors

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Keywords

Menidia conchorum, bottleneck, inbreeding, Florida Keys

Abstract

Species diversity in fish is higher in freshwater than in the marine environment, suggesting that diversification rates are higher among freshwater fish. This has been empirically demonstrated in New World silversides (Teleostei, Atherinopsidae), a group of small, surface-dwelling fish comprised of both freshwater and marine species. The mechanism underpinning elevated diversification in freshwater is proposed to be that freshwater fish populations are more fragmented than marine populations, promoting more rapid genetic diversification due to genetic drift and local selection acting on small, isolated gene pools. To test this hypothesis on a microevolutionary scale we examined the population genetics of *Menidia conchorum*, a New World silverside that is endemic to the Florida Keys and inhabits small, tidally restricted hypersaline pools. It is an inland ecotype of the more widespread coastal species *Menidia peninsulae* found along the coast of Florida and the Gulf of Mexico. We compared genetic diversity and effective population size N_e of these two species and tested for inbreeding and bottlenecks. In seven pools inhabited by *M. conchorum* we also measured changes in genetic diversity over 13 generations. Our findings indicate that *M. conchorum* exist as a series of isolated, fragmented populations in the Florida Keys while their parent species in the marine environment is more genetically diverse and less structured. We found that genetic diversity and N_e decreased over time in six of seven pools sampled 13 generations apart, while population differentiation and inbreeding increased. These findings are consistent with the hypothesis that the fragmented nature of inland bodies of water promotes rapid genetic change and diversification among species that live within them.

Title: Genetic estimation of effective population size and detection of recent bottlenecks and inbreeding in marine fish populations: a review.

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Keywords

Effective number of breeders, genetic diversity, null alleles, N_c/N_e ratio

Abstract

The effective population size (N_e) is the number of reproductively mature adults effectively contributing to the next generation. Genetic drift and inbreeding, two primary forces driving the loss of genetic diversity in populations, increase as N_e decreases. A sudden, severe population collapse (bottleneck) can also result in the loss of genetic diversity. Marine fish populations are generally considered to be large, panmictic and thus resistant to the erosion of genetic diversity through these processes. Recent studies, however, indicate that marine fish populations are frequently more structured than previously thought and N_e can be up to five orders of magnitude smaller than census population size. To assess the risks of genetic diversity loss to marine fish populations, I reviewed the literature ($N=53$ studies) on N_e , inbreeding and bottlenecks in this group. Though study species covered a large taxonomic range (16 orders) and trophic levels, there was a strong bias towards commercial species and populations in the Atlantic. While reported N_e estimates ranged from 10 to $>10^6$ individuals, approximately half were below critical population sizes for which loss of adaptive potential and/or inbreeding can be a concern and only 21% of populations had sufficient N_e to maintain long-term stability based on commonly used thresholds. Bottlenecks were detected in approximately half of the populations tested; though in some cases known demographic bottlenecks did not result in a detectable genetic bottleneck. Only 6 studies reported evidence of inbreeding in marine fish, but relatively few studies explicitly test for it and so it may be underestimated. My review suggests that marine fish can be vulnerable to a range of processes that affect their genetic diversity and this needs to be better incorporated into management and conservation. I also suggest N_e and systematic testing for bottlenecks and inbreeding should be a staple of marine fish population genetic studies and reported transparently using consistent, specific terminology.

Published:

Title: Novel microsatellite loci for winter flounder (*Pseudopleuronectes americanus*)

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Abstract

Since the 1980s, heavy exploitation has led to severe population declines in winter flounder, *Pseudopleuronectes americanus*, a demersal flatfish inhabiting the inshore bays and estuaries of the Northwest Atlantic. Recent studies using a limited number of non-specific microsatellite loci have demonstrated fine-scale population structure. Here, we present 21 novel species-specific microsatellite loci to enhance studies of genetic diversity, population differentiation, effective population size and mating behavior of this important commercial species.

Title: Novel microsatellite loci for white, *Carcharodon carcharias* and sandtiger sharks, *Carcharias taurus* (Order Lamniformes).

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Abstract

The white shark, *Carcharodon carcharias*, and sand tiger shark, *Carcharias taurus*, are large-bodied lamniform sharks that are apex predators worldwide throughout the temperate zone. Both species have very low rate of intrinsic population increase and are of conservation concern due to past or present overexploitation. So far only a few microsatellite loci have been developed for population genetics studies on these sharks. Here we present ten novel loci for white sharks and five novel loci for sand tigers. These markers will enable higher resolution population genetic studies and open the possibility of comprehensive studies of individual relatedness and breeding biology.

Title: Severe inbreeding and small effective number of breeders in a formerly abundant marine fish.

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Abstract

In contrast to freshwater fish it is presumed that marine fish are unlikely to spawn with close relatives due to the dilution effect of large breeding populations and their propensity for movement and reproductive mixing. Inbreeding is therefore not typically a focal concern of marine fish management. We measured the effective number of breeders in 6 New York estuaries for winter flounder (*Pseudopleuronectes americanus*), a formerly abundant fish, using 11 microsatellite markers (6-56 alleles per locus). The effective number of breeders for 1-2 years was remarkably small, with point estimates ranging from 65-289 individuals. Excess homozygosity was detected at 10 loci in all bays ($F_{IS} = 0.169-0.283$) and individuals exhibited high average internal relatedness (IR; mean = 0.226). These both indicate that inbreeding is very common in all bays, after testing for and ruling out alternative explanations such as technical and sampling artifacts. This study demonstrates that even historically common marine fish can be prone to inbreeding, a factor that should be considered in fisheries management and conservation plans.